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ANALYSES FOR CERTAIN AMINO-ACIDS IN TUBERCULIN PROTEIN FRACTIONS.

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PROTEIN fractions with contrasting physico-chemical and biological properties have been isolated from unheated culture filtrates of tubercle bacilli grown upon a non-protein synthetic medium. Two of these fractions isolated from the filtrate of a single strain as well as from different strains and containing proteins with widely differing properties were chosen, even though they were not molecularly homogeneous, for a preliminary study of some of their amino-acids.

These were the proteins designated as A and C proteins (Seibert, 1949). Their known contrasting properties are as follows: The A protein was colourless in a 1 per cent concentration; it travelled in electrophoresis with the slowest mobility, about -3 to -4×10^{-5} cm.^{2}/volt^{-1}/sec.$^{-1}$, in phosphate buffer pH 7·0, $\mu = 0{\cdot}1$; it was soluble at its isoelectric point; it was associated with a considerable amount of polysaccharide material, and it was the most potent fraction per milligram of nitrogen in the tuberculin skin test, in producing lethal reactions in tuberculous animals and in eliciting the Arthus reaction in normal animals. The C protein gave a deep amber solution in 1 per cent concentration; it was associated with very little carbohydrate material; it was insoluble at its isoelectric point; it had a much faster electrophoretic mobility ($-6{\cdot}0$ to $-7{\cdot}0 \times 10^{-5}$/cm.^{2}/volt^{-1}/sec.$^{-1}$) than the A protein, and was the least potent in the biological properties enumerated under the A protein. The ultraviolet absorption characteristics of the A and C proteins differed chiefly at a wave-length of 2500 Å (Seibert, 1949).

These variations in physico-chemical properties would be consistent with differences in the amino-acid composition, particularly the aromatic amino-acids, of the A and C proteins. The object of the present work is an investigation of the possibility of such differences, and an attempt to correlate the results with the biological activities of tuberculin proteins.

EXPERIMENTAL.

The average tuberculin potencies of a number of the A and C proteins tested simultaneously in 6 to 8 tuberculous guinea-pigs in each series against a constant standard purified protein derivative (PPD-S) are given in Table I. The sizes of

the skin reactions were measured in two diameters at right angles to each other and averaged ; the first figures in each reaction measurement are recorded in the table. The height of induration is given in the second figure. The averages of such measurements in 6 to 8 guinea-pigs in each case are given. The reactions at 48 hours to the specified fraction and the corresponding reactions with the standard on the same guinea-pigs are shown, and differences in the sizes of these two reactions appear in the last column.

A general survey of the amino-acid compositions of these proteins was first made by means of paper chromatographic analyses. The most favourable conditions for complete hydrolysis were found to be with 5N HCl in sealed tubes at 110° C. for 17 hours. The technique of Consden, Gordon and Martin (1944) was used, with butanol-water as the eluting system in the first dimension. For the second dimension, phenol-water ascending the chromatogram (Williams and Kirby, 1948) was used.

TABLE I.—*Tuberculin Potencies of A and C Tuberculin Proteins.*

Fraction.	Lot number.	Average size skin reactions in mm. to— Fraction.	Standard (PPD–S).	Differences in sizes of reactions.
A	96 A IVb	19·8 × 3·0	16·8 × 3·0	+ 3·0 × 0
	96 B III	19·8 × 3·0	14·6 × 2·3	+ 5·2 × 0·7
	97 A	18·5 × 2·8	16·1 × 2·5	+ 2·4 × 0·3
	98 III	23·6 × 3·7	18·0 × 2·6	+ 5·6 × 1·1
C	96 A I	18·0 × 2·7	21·9 × 3·3	− 3·9 × 0·6
	98 I	15·8 × 2·5	16·9 × 3·0	− 1·1 × 0·5
	100 I	18·0 × 2·3	19·0 × 2·9	− 1·0 × 0·5

The following amino-acids appeared to be present in two different A and one C fractions analysed : aspartic or glutamic acid or cystine, glycine, arginine, lysine, valine, phenylalanine, tyrosine, alanine, and tryptophan. There were present also two unidentified spots, possibly di-or tripeptides.

Quantitative chemical analyses were then made of the contents of the phenolic groups by the Folin-Ciocalteu (1927) reagent. Tryptophan gives 89 per cent of the colour given by tyrosine by this method. Tryptophan was also estimated directly by the method of Bates (1937). Analyses were made on four A fractions and four C fractions.

Filtered solutions of all these preparations were analysed for their nitrogen content, from which the total protein concentrations were calculated. From these same solutions aliquot 0·2 ml. quantities were mixed with 0·2 ml. concentrated HCl in sealed tubes and hydrolysed for 17 hours at 110° C. The hydrolysed solutions were made to 5 ml. with water, centrifuged free of humin, and analysed for their content of phenolic groups. Pure tyrosine is not destroyed under these conditions of hydrolysis, but tryptophan is to the extent of about 28 per cent. Other aliquots of the original unhydrolysed solutions were analysed directly for their content of phenolic groups and of tryptophan. The results were then calculated as per cent of the protein present as phenol groups (using tyrosine as a standard substance) or tryptophan, and are shown in Table II.

The results show that the tryptophan content of the A protein fractions was higher than that of the C protein fractions. With one exception the content of

phenolic groups was also higher in the unhydrolysed A fractions than in the C fractions. On hydrolysis, still more phenolic groups were found in the A fractions, whereas no extra ones appeared in the C fractions. It seems probable, therefore, that some phenolic groups in the A fractions become unmasked during hydrolysis.

TABLE II.—*Analyses of Phenolic Groups and Tryptophan in A and C Tuberculin Proteins.*

Type of protein.	Lot number.	Strain of tubercle bacillus.	Per cent of protein as— Tryptophan. Unhydrolyzed protein.	Phenolic groups. Unhydrolyzed protein.	Phenolic groups. Hydrolyzed protein.
A	96 A IVb	A 33	4·6	5·7	6·3
	96 B III	A 33	4·6	5·5	6·6
	97 A	DT	3·6	5·2	6·8
	98 III	DT	3·4	4·6	7·6
C	95 I	BCG	2·2	4·4	4·4
	96 A I	A 33	1·9	4·5	4·6
	98 I	DT	2·0	3·8	3·8
	100 I	H 37	2·6	4·7	4·9

DISCUSSION.

Considerable quantities of carbohydrate have been found in all the A protein fractions in their present stage of purity, and while some of the carbohydrate migrates independently in electrophoresis and can be easily removed by chemical means, another portion seems to be combined by a stronger chemical bond. This may account for the fact that the A protein fractions have on occasions exhibited less stability in their solubilities as well as in their potencies.

Since the A protein fractions are the most potent ones biologically, as mentioned above, it is tempting to speculate that the higher content of tryptophan or of the masked phenolic groups may be of importance in helping to determine the biological potencies. Association between biological potency and the content of phenolic groups has been demonstrated for a number of other proteins, such as pepsin by Herriott (1935), papain by Fruton and Lavin (1939), insulin by Crammer and Neuberger (1943), and tobacco mosaic virus by Miller (1942). Fernbach and Rullier (1940), however, did not find a strict parallelism between the potency of several of their tuberculin preparations and the extent to which these reacted with the Millon reagent.

On the other hand, Kallos (1933) suggested that tuberculin potency paralleled the content of tryptophan in his preparations.

SUMMARY.

At least ten of the ordinary amino-acids have been found by paper chromatography in two tuberculin protein fractions A and C, isolated from unheated culture filtrates of tubercle bacilli grown on synthetic medium.

More tryptophan and phenolic groups were found in the unhydrolysed A than

in the C fractions. Still more phenolic groups were found after hydrolysis in the A fractions, but not in the C fractions.

Since the A fractions are more potent biologically than the C fractions a possible connection between the presence of these groups and potency is suggested.

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AN Rh ALLELE ON THE E-e LOCUS REACTING WITH BOTH ANTI-E AND ANTI-e SERA.

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A NUMBER of allelomorphs of the Rh genes on the loci C-c, D-d and E-e have been described. Stratton (1946) described an allele of D which he designated D^u, Callender and Race (1946) identified an allele of C which they named C^w, and Race, Sanger and Lawler (1948*a*, *b*) described two further alleles of C which they called C^u and c^v respectively. Recently Ceppellini, Ikin and Mourant (1950) have described an allele of E which bears a close resemblance in its reactions with anti-E sera to the reactions of the alleles D^u and C^u with anti-D and anti-C sera respectively.

The purpose of this paper is to describe a further allele of E which appears to be capable of reacting with both anti-E and anti-e sera.

The new allele was first detected early in 1948 as an apparent anomaly in a family pedigree. G. L—, the child of a multiparous woman, Mrs. L—, was typed for its A-B-O and Rh group at delivery, and was found to belong to group A_1 cdE/cde. The direct Coombs test on the baby's cells was negative. Investigation of Mrs. L— showed her to belong to group A_1 cde/cde, and to have a mixture of anti D + E agglutinins in her serum. Her previous history indicated that during earlier pregnancies she had had a number of transfusions of blood of unknown Rh type. Soon after her delivery the husband was grouped and Rh typed in order to determine whether he was homo- or heterozygous, and he appeared to type as CDe/cde. In an attempt to explain the apparent anomaly in the Rh types of the parents and the child G. L— further investigations were carried out on the family, including the other surviving child, S. L—, using an absorption technique. It was found that although the cells of neither Mr. L— nor S. L— could be agglutinated by anti-E sera, they were capable of effecting a marked absorption of these anti-E sera, while CDe/CDe and cde/cde cells used as controls left the anti-E sera completely unabsorbed. Two years after the initial experiments were carried out the whole family was re-investigated, using more extensive methods and a larger panel of anti-E sera.

EXPERIMENTAL.

The serological investigation of this family consisted of the following:

1. Direct typing with nine anti-E sera of Mr. L—, Mrs. L—, S. L— and G. L—, using saline and albumen as diluents.

2. Attempted sensitization of the cells of Mr. L—, Mrs. L— and S. L—, with eight anti-E sera for testing by the indirect Coombs test.

3. Attempted agglutination of the cells of Mr. L—, Mrs. L— and S. L— with seven anti-E sera by means of the trypsinization technique of Pickles (1950).

4. Absorption experiments with three anti-E sera using the cells of Mr. L— and S. L— for absorption.

5. Experiments showing the effect of absorption of anti-E sera by the cells of Mr. L— and S. L—, on the values obtained when the cells thus treated with anti-E sera were used in the titration of an anti-e serum.

6. Experiments in the elution of anti-E agglutinin absorbed on to the cells of Mr. L— and S. L—.

7. Typing of the family for all the blood group systems for which anti-sera were available in an attempt to establish the relationship between Mr. L— and G. L—.

Direct typing of Mr. L—, Mrs. L—, S. L— and G. L—, with anti-E sera.

For the direct typing experiments nine anti-E sera were used, all these sera but one (anti-E (9)) being controlled for the presence of contaminating incomplete anti-D by testing them against cDe cells in saline, albumen, and by the indirect Coombs test and the trypsin technique.

All these controls were completely negative.

The tests were carried out by the usual tube technique, using 3 per cent suspensions of the cells under test in saline and in 30 per cent bovine albumen, and incubating for 4 hours at 37° C.

Mr. L—'s cells and Mrs. L—'s cells gave negative results with all the sera in both saline and albumen, S. L—'s cells gave negative results with all the sera in saline and with all save one (anti-E (7)) in albumen, while G. L—'s cells gave strongly positive agglutinations with all the sera in both saline and albumen (Table Ia).

Indirect anti-human-globulin tests on the cells of Mr. L—, Mrs. L— and S. L— after attempted sensitization with anti-E sera.

An attempt to sensitize these cells was made with eight of the anti-E sera used in the previous experiments.

TABLE Ia.—*Cells of Mr. L—, Mrs. L—, —, S. L— and G. L— in Saline and Albumen Diluents with Anti-E Sera.*

Anti-E.	Diluent saline cells. Mr. L—.	Mrs. L—.	S. L—.	G. L—.	R_1r.	R_2r.	R_0.	rr.	Diluent 30% bovine albumen cells. Mr. L—.	Mrs. L—.	S. L—.	G. L—.	R_1r.	R_2r.	R_0.	rr.
(1)	—	—	—	V	—	V	—	—	—	—	—	V	—	V	—	—
(2)	—	—	—	V	—	V	—	—	—	—	—	V	—	V	—	—
(3)	—	—	—	V	—	V	—	—	—	—	—	V	—	V	—	—
(4)	—	—	—	V	—	C	—	—	—	—	—	V	—	C	—	—
(5)	—	—	—	V	—	C	—	—	—	—	—	V	—	C	—	—
(6)	—	—	—	V	—	V	—	—	—	—	—	V	—	C	—	—
(7)	—	—	—	V	—	V	—	—	—	—	+	V	—	C	—	—
(8)	—	—	—	V	—	V	—	—	—	—	—	V.	—	V	—	—
(9)	—	—	—	V	—	±	..	—								

The reactions given in this and subsequent tables are as follows: C, complete agglutination; V, visual agglutination; ±, large clumps of cells; +, smaller clumps of cells; —, no agglutination.

Three per cent suspensions of cells from Mr. L—, Mrs. L— and S. L— were incubated with the anti-E sera for 4 hours at 37° C., washed twice in a large bulk of saline and exposed to anti-human-globulin rabbit serum. All these tests gave negative results (Table Ib).

Effect of trypsinization on the agglutination of cells from Mr. L—, Mrs. L— and S. L— with anti-E sera.

The effect of trypsinization on these cells was studied using the technique recommended by Pickles (1950).

The cells were exposed to the action of the buffered trypsin solution for 30 minutes at 37° C. and washed once with saline. One volume of a 5 per cent suspension of the trypsinized cells was then added to the anti-E serum under test, and the mixture was incubated for 1 hour at 37° C. The tests were controlled by using CDe/cde and cde/cde cells, trypsinized, washed and incubated in the same way with the anti-E sera. After trypsinization, the control cells and cells from Mrs. L— showed no agglutination with the anti-E sera tested, but Mr. L—'s cells showed positive agglutination with three, and S. L—'s cells showed positive agglutination with five out of the seven anti-E sera used in the tests (Table Ib).

TABLE Ib.—*Effects of the Indirect Coombs Test and the Trypsin Test on the Cells of Mr. L—, Mrs. L— and S. L—.*

	Indirect Coombs test cells.			Trypsin test cells.				
Anti-E.	Mr. L—.	Mrs. L—.	S. L—.	Mr. L—.	Mrs. L—.	S. L—.	R_1r.	rr.
(1)	—	—	—	—	—	V	—	—
(2)	—	—	—	—	—	—	—	—
(3)	—	—	—	—	—	—	—	—
(4)	—	—	—	∓	—	V	—	—
(5)	—	—	—	—	—	V	—	—
(6)	—	—	—	∓	—	∓	—	—
(7)	—	—	—	V	—	V	—	—
(8)	—	—	—	..	..	..	..	..
(9)	..	..	..	..	..	..	..	..

Absorption of anti-E sera with cells from Mr. L— and S. L—.

A series of absorption experiments were carried out on the cells of Mr. L— and S. L—, using five anti-E sera, including three powerful ones (anti-E's (3), (4) and (5)), in order to confirm the results obtained two years earlier.

The cells of Mr. L— and S. L— were washed and packed by centrifuging, and equal volumes of the packed cells and serum under test were mixed and incubated at 37° C. for 4 hours. After centrifuging, the supernatant was removed and titrated against cells of type cDE/cde.

Non-specific absorption was controlled by incubating packed, washed CDe/cde cells with the anti-E sera under test for 4 hours, centrifuging and titrating the supernatant with the cDE/cde cells.

The initial titres of the unabsorbed anti-E sera were determined by titration with the same cDE/cde cells.

Significant absorption of the anti-E from four out of the five sera was obtained with both Mr. L—'s and S. L—'s cells. There was no absorption of the anti-E sera with CDe/cde cells (Table II).

TABLE II.—*Absorption of Anti-E Sera with Cells Mr. L— and S. L—.*

Anti-E sera.	Titre after absorption with cells.			Titre of unabsorbed serum.
	Mr. L—.	S. L—.	CDe/cde.	
(1)	1	1	8	8
(2)	1	1	4	4
(3)	16	16	32	32
(4)	2	4	32	32
(5)	4	4	32	32

Titration of Mr. L—'s, S. L—'s and G. L—'s cells with anti-e serum.

As the allele of E present in Mr. L— and S. L— was so weak in its reactions with anti-E sera, the cells of Mr. L— and S. L— were titrated with an anti-e serum to see whether they gave a single or double dose reaction, the cells of G. L— also being titrated with the anti-e serum in order to see if they gave the expected single dose reaction. The anti-e serum was titrated against cde/cde cells as the double dose and cDE/cde cells as the single dose control. The cells of Mr. L— and S. L— gave a full double dose reaction with the anti-e serum and the cells of G. L— gave the expected single dose reaction (Table III).

The effect of incubating the cells of Mr. L— and S. L— with anti-E serum on the double dose reaction with anti-e serum.

As the cells of Mr. L— and S. L— were inagglutinable with anti-E sera in saline, it was decided to try the effect of incubating these cells with powerful anti-E sera, washing them free from anti-E and then using them to titrate the anti-e serum.

Washed, packed cells from Mr. L— and S. L— were absorbed with three powerful anti-E sera (anti-E's (3), (4) and (5)) for 4 hours at 37° C., washed free from anti-E serum and made into 3 per cent suspensions in saline. The anti-e serum was then titrated against these suspensions. As controls the anti-e serum was titrated against untreated cells from Mr. L— and S. L—, and also against cells of types cDE/cde and cde/cde. It was found that the effect of the treatment of the cells of Mr. L— and S. L— with anti-E sera was to reduce their previous double dose reaction with anti-e serum to a single dose reaction. To exclude this phenomenon being due to a non-specific absorption further controls were carried out by incubating CDe/cde and cde/cde cells with anti-E sera for 4 hours at 37° C., washing and using these cells to titrate the anti-e serum.

After treatment with the anti-E sera, the CDe/cde and cde/cde cells still gave a full double dose reaction with the anti-e serum, indicating that the absorption

TABLE III.—*Titration of Anti-e Serum with Cells of Mr. L— and S. L— Before and After Incubation with Anti-E Serum.*

Cells.	Titre of anti-e serum using *untreated* cells.	Titre of anti-e serum using test cells which have been incubated with anti-E serum.		
		Anti-E (3).	Anti-E (4).	Anti-E (5).
Mr. L—.	64	8	8	8
S. L—	64	16	16	16
G. L—	8	(Cells agglutinated by anti-E)		
cde/cde	64	64	64	64
CDe/cde	64	64	64	64
cDE/cde	8	(Cells agglutinated by anti-E)		

of anti-E in the case of the cells of Mr. L— and S. L— was specific in nature (Table III).

It will be seen from Tables II and III that although anti-E (3) was apparently absorbed on to the cells of Mr. L— and S. L— sufficiently to suppress the reaction with the anti-e serum, it did not give a significant absorption value.

Elution of anti-E agglutinin from the cells of Mr. L— and S. L—.

Elution experiments were carried out to show that anti-E agglutinin was present on the cells of Mr. L— and S. L— after these cells had been used to absorb anti-E sera, and to confirm the anti-E specificity of these cells as demonstrated by the absorption and trypsin tests.

The packed, washed cells from Mr. L— and S. L— were incubated for 4 hours at 37° C. in the proportions of 0·5 c.c. packed cells to 0·5 c.c. of anti-E serum with two anti-E sera (anti-E's (4) and (5)), both of which gave good absorption values.

The cells were then centrifuged and the supernatant serum removed. The cells were washed twice in a large bulk of saline to remove residual anti-E serum, the efficiency of the washing being controlled by incubating a volume of saline from the second washing with a 3 per cent suspension of cdE/cde cells in saline for 4 hours at 37° C. The washed, packed cells were then diluted with 0·5 c.c. of saline and heated with constant agitation in a water-bath, at 56°C. for 5 minutes. The tests were removed from the water-bath, placed in the centrifuge with the centrifuge buckets containing water heated to 56° C. and centrifuged hard for 2 minutes.

The supernatant was then quickly removed.

The supernatant eluates were then tested for the presence of anti-E agglutinins against cells of type cdE/cde and controlled for non-specific reactions by testing against cells of type CDe/cde and cde/cde, by incubating a volume of the eluate with a volume of 3 per cent saline suspension of the cells under test. Positive reactions were obtained with the eluates from the cells of Mr. L— and S. L— against cells of type cdE/cde, while the control tests with cells of type CDe/cde and cde/cde were negative (Table IV).

TABLE IV.—*Elution of Anti-E Agglutinins from Cells of Mr. L— and S. L—*

Test cells.	Eluate from cells Mr. L—.		Control saline from second washing.		Eluate from cells S. L—.		Control saline from second washing.	
	Anti-E (4).	Anti-E (5).	Anti-E (4).	Anti-E (5).	Anti-E (4).	Anti-E (5).	Anti-E (4).	Anti-E (5).
cdE/cde	+	+	−	−	+	+	−	−
CDe/cde	−	−	−	−	−	−	−	−
cde/cde	−	−	−	−	−	−	−	−

Blood group investigations on Mr. L—, Mrs. L—, S. L— and G. L—.

These investigations were carried out in order to establish as far as is possible by these methods the relationship between Mr. L— and G. L—. The whole family was typed for the following blood group systems:

A-B-O-, M-N-S, Rh, P, Le, Lu, K and Fy.

The only discrepancy demonstrable by this investigation was the difference in

the reactions given by the cells of Mr. L— and G. L— with the anti-E and anti-e sera.

The results of this investigation are shown in Fig. 1, in which the allele on the E-e locus in the cases of Mr. L— and S. L— is shown as e^x for reasons which will be given in the discussion of these results.

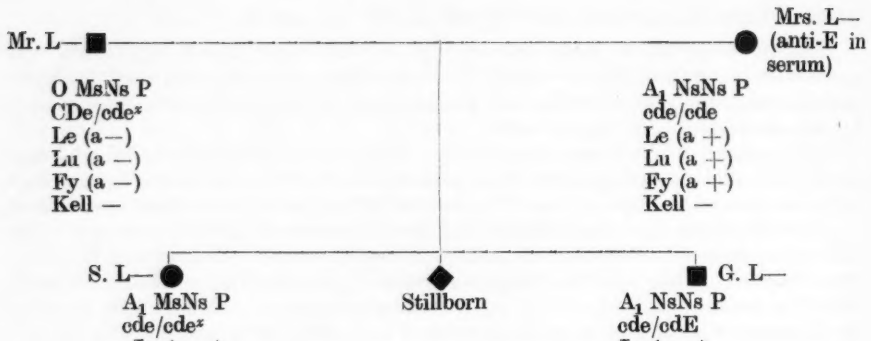

FIG. 1.—Blood group pedigree of the " L " Family.

Note.—When typed with anti-Le^a serum at birth, the child G. L— typed as Le(*a*+). When retyped in the present series of experiments at the age of two years he typed as Le (a—).

DISCUSSION.

The foregoing experiments show that in the cases of two members of the " L " family, Mr. L— and S. L—, the E-e locus on the cdE chromosome is occupied by an allele of E which reacts both with anti-E and anti-e sera.

The ability of cells from Mr. L— and S. L— to react with anti-E sera is shown by the absorption and elution experiments, the experiments using the trypsin technique by which means agglutination of the cells with certain anti-E sera could be obtained, and by the suppressive effect of the incubation of cells from Mr. L— and S. L— with anti-E sera on the subsequent reactions of these cells with anti-e serum.

That the cells from Mr. L— and S. L— react with anti-e sera is shown by the double dose reaction given by these cells on titration with anti-e serum.

The experimental results do not yield sufficient information to make it possible to come to any conclusions regarding the mechanism governing these reactions, but the answer may possibly be found in the theory put forward by Race, Sanger and Lawler (1948*b*) to account for similar dual reactions by the allele c^v. These authors postulate that the reaction of c^v with anti-C sera was due to the fact that the anti-C sera were of specificity anti-C + c^v. The allele of E found in the " L " family differs from the corresponding allele c^v in that while c^v gave direct agglutination with anti-C sera the allele of E is agglutinated only after trypsinization, and is otherwise detected only by an absorption technique.

An alternative theory is that in the case of this E allele, the red cells have the two antigenic components E and e lying close together, with the e antigen partially " overshadowing " the E. This would account for the double dose reaction with anti-e serum, and could be made to account for the ability of the allele to absorb anti-E sera if it is assumed that as the " overshadowing " is only partial there is sufficient E antigen available to absorb anti-E sera, but insufficient to allow agglutination to take place.

This theory is further supported by the effect of trypsinization, by which the E antigen is rendered agglutinable. This may be due to the trypsin reducing the degree of overshadowing of E by e, and thus making more E antigen available to the antibody.

From the reactions given it will be seen that although this E allele is similar in its reactions with anti-E and anti-e sera to c^v with anti-C and anti-c sera, it differs in the important factor of its inagglutinability other than after trypsinization. Its reactions are such as to suggest that it is more closely related to e than E, and it is therefore proposed to call it e^x.

The finding that incubation of cells from Mr. L— and S. L— with anti-E sera had the effect of partially suppressing the reaction of these cells with anti-e serum, reducing the previous double dose reaction to a single dose reaction, is of considerable interest. In connection with this finding it is worthy of note that one anti-E serum (anti-E (3)), while not giving a significant absorption value with these cells, nevertheless had a marked suppressive effect on the subsequent reactions with anti-e serum equal to the suppression obtained with anti-E's (4) and (5), both of which gave good absorption values.

As has been pointed out, the evidence available is insufficient to allow of conclusions regarding the mechanism of these reactions, and this observation is therefore presented without comment.

The presence of E in its normal agglutinating form in G. L— is of considerable interest. That there is a relationship between the E present in G. L— and the e^x present in Mr. L— and S. L— seems to be in little doubt. It will be seen from the blood group pedigree that this constitutes the only anomaly, and it is submitted that this degree of correlation between eight separate blood group systems plus the fact that although the cdE chromosome is of low frequency in England it exists in the normal form in G. L— and in the modified form cde^x in Mr. L— and S. L—, is such as to show a very high probability of relationship between the chromosome cdE in G. L— and the chromosome cde^x in Mr. L—.

It may be offered as an objection that false positive reactions with anti-E sera are sometimes obtained with the cells of new-born infants. It will, however, be noted that G. L— still gave strongly positive reactions with anti-E sera at the age of 2 years.

A point of great importance which arises from these findings is the fact that the allele of E present in Mr. L— was not detected by agglutination methods of the type normally employed. This has considerable bearing on the question of the use of the Rh system in medico-legal blood group determinations. By the methods usually employed in Rh testing, Mr. L— would have been typed as CDe/cde, and the implications would have been obvious.

The fact that he had on the E-e locus a modification of E was demonstrated only by the use of specialized techniques. Such alleles must be very rare, and are difficult to account for. It may be that the modified form of E in Mr. L—

and S. L— is due to some abnormality of the cell which is not present in G. L—, or it is possible that in G. L— there has been a mutation from e^x to E. There is no experimental evidence for either theory, but that such alleles exist should be kept in mind in view of the serious nature of the evidence provided by blood groups in medico-legal work.

SUMMARY.

1. An allele of E reacting with both anti-E and anti-e sera is described.
2. The allele gives certain reactions analogous to c^v, but differs in its powers of agglutinating.
3. Possible reasons for the reactions of the allele with anti-E and anti-e sera are discussed.
4. The presence of the E antigen in the normal form in the second child is discussed, together with the possibility of similar cases arising in medico-legal blood grouping.

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THE EFFECTS OF BIOCHEMICAL MUTATION ON THE VIRULENCE OF *BACTERIUM TYPHOSUM*: THE INDUCTION AND ISOLATION OF MUTANTS.

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THE outcome of the infection of a particular host by a particular parasite is recognized to be dependent upon a number of variable factors. Alteration of one of these in either the infecting organism or in the infected host may consequently be expected to alter the outcome of that particular host-parasite relationship. It is evident that the synthetic processes involved in the elaboration of of the bacterial body must play a primary role in determining the properties of the mature organism and its fate on introduction into a host and the interference with such processes may well result in alterations of one or more factors affecting this relationship. It was considered of interest to investigate to what extent such interference occasioned by the mutational blockage of specific synthetic enzyme systems would be reflected in an alteration of the virulence of a pathogenic organism. The typhoid organism was shown by Gladstone (1937) to be fully virulent for mice when grown on a simple synthetic medium, and was considered to be suitable for a study of this nature.

A number of agents capable of inducing mutations with respect to the synthetic abilities of bacteria are known. Those most commonly employed are X-rays, ultraviolet light and the nitrogen and sulphur mustards. The precise mode of action of these mutagenic agents is not fully understood, but their final effects are attributed to the inactivation of inheritable determinants governing the production or specificity of enzyme systems necessary for the synthesis of essential metabolites. To permit the growth of mutant forms these essential metabolites, or growth factors, must be added preformed to a medium. In current terminology forms differing from the parent in requiring added growth factors are " biochemical " mutants.

Induced biochemical mutation in *Bact. typhosum* has been investigated by few authors. Grainger (1947) was unable to select from 1000 isolates tested any mutants differing from the parent culture in colony morphology fermentation or biochemical reactions after exposure to X-rays. Following culture in the presence of acenaphthene, Grainger and Wilmer (1947) selected a rough variant unable to ferment galactose. Kristensen (1948), by serial transfer demonstrated the ability of his strains to mutate with respect to their fermentation of various sugars. Strains of typhoid bacteria were shown by Fildes and Whitaker (1948) and Mercedes (1948) to mutate spontaneously at a low rate, from tryptophan dependence to independence.

The method of isolating mutants with decreased synthetic ability initiated by Beadle and Tatum (1941) in their pioneer work on *Neurospora* is applicable to the isolation of similar mutants of bacteria. However, it is limited in that comparatively small numbers of a mixed population of mutant and predominantly non-mutant forms can be conveniently examined. Various mitigating procedures have been described by Lederberg and Tatum (1946), Farghaly, Miller and McElroy (1948), Miller, Farghaly and McElroy (1949), Meyersberg, Pomper and Cutter (1949) and Davis (1949*b*). A notable improvement simultaneously published by Davis (1948), and Lederberg and Zinder (1948) utilizes the selective bactericidal action of penicillin on growing cells whilst leaving non-growing cells without permanent effect for the selection of biochemical mutants from very large mixed populations.

The present paper describes the methods used for induction and selection of biochemical mutants of *Bact. typhosum*.

MATERIALS.

Organism.

The strain Ty2 described by Felix (1938) and employed by Henderson (1939) was used as starting material.

Maintenance of cultures.

All cultures of parent and mutants were dried (Stamp, 1947) from growth on minimal medium supplemented by growth factors as necessary, and stored at $\pm$ 2° C. *in vacuo*.

Suspensions.

Dried cultures were emulsified in tryptic meat digest broth (TMB) and plated on tryptic meat digest agar (TMA). After overnight incubation, slopes of minimal medium supplemented as necessary were inoculated from these plates. After 18 hours' incubation the slopes were emulsified in phosphate buffer pH 7·4 (KH_2PO_4 4·5 g./litre + NaOH to pH 7·4) to the desired opacity and the suspensions obtained used without further treatment. Fresh dried cultures were used for each suspension prepared on different days to avoid subculturing with the accompanying possibility of variation.

Incubation.

Cultures were incubated unshaken at 37° C. in an atmosphere of approximately 5 per cent CO_2: 95 per cent air (Gladstone, Fildes and Richardson, 1935).

Minimal medium.

It was essentially that used by Gladstone (1937) and had the following composition:

1. KH_2PO_4 4·5 g., NH_4Cl 0·5 g., $(NH_4)_2SO_4$ 0·5 g., *ca* N/1 NaOH 24 ml. (to pH 7·4). Water 950 ml.
2. $MgSO_4.7H_2O$ 0·037 g. Water 10 ml.
3. Glucose 2·25 g. Water 20 ml.

Solutions 1 and 2 were autoclaved at 115° C. for 20 minutes in separate vessels, and 3 was Seitz filtered. Solutions 2 and 3 were added to 1 when cool and the mixture dispensed aseptically into tubes 11·0 cm. × 1·5 cm., using an automatic filler delivering 6 ml. to each tube. As necessary the medium was solidified with 1 per cent agar autoclaved separately. The customary precautions regarding purity of chemicals and cleanliness of glassware were observed throughout.

Complete medium.

Tryptic meat digest formed the basis of the complete medium. This was supplemented by the addition of 1 g. Difco yeast per litre and later further supplemented with purines and pyrimidines, each to a concentration of 50 μM.

Growth factors.

The following were held as stock solutions:

Glycine.	L (−) Cystine.	Aneurin.	Adenine.
Alanine.	Methionine.	Riboflavin.	Guanine.
Valine.	Aspartic acid.	Nicotinamide.	Xanthine
Leucine.	Glutamic acid.	Ca. pantothenate.	Hypoxanthine.
Isoleucine.	Lysine.	Pyridoxin.	Cytosine.
Norleucine	L (+) Arginine.	Choline chloride.	Thymine.
Proline.	L (−) Histidine.	Inositol.	Uracil.
L (−) Hydroxyproline.	Phenylalanine.	Biotin concentrate.	Ribose.
Serine.	Tyrosine.	Pimelic acid.	Haemin.
Threonine.	Tryptophan.	Para amino benzoic acid.	Glutamine.
		Folic acid.	Oleic acid.

Except where indicated the amino acids were racemic mixtures. The L-isomers were thrice recrystallized from suitable solvents.

EXPERIMENTAL AND RESULTS.

1. *Selection of ammonia assimilating strain of Ty2.*

A dried culture of Ty2 was four times selected from single colonies on TMA and confirmed as consistent with *Bact. typhosum* in its sugar reactions and agglutination by typhoid Vi, O and H antisera. Visible growth occurred in 66 hours following the inoculation of *ca.* 5×10^5 twice washed cells into minimal medium, both with and without added tryptophan (100 μM final concentration). Twelve serial transfers were made in minimal medium and the resulting culture four times selected from single colonies on minimal medium agar. The final culture grown on TMA resembled the original Ty2 in all respects. Loss of H antigen occurred when grown on minimal medium, as previously noted by Gladstone (1937). A considerable number of dried cultures, sufficient to outlast this study, were prepared and the strain, designated Ty22, used for all subsequent experiments.

2. *Induction of mutants.*

(a) *By X-irradiation.*—An apparatus manufactured by the Picker X-ray Corporation was employed and operated at 60 peak kilovolts with a tube current of 4 milliamperes. Five ml. of a suspension with 2×10^9 organisms per ml. was

contained in a sterile pyrex chamber 2·5 cm. diameter and 5 cm. deep in which a platinum stirrer, glass cooling coil and thermocouple were incorporated. The chamber was located vertically above the tube with its base 5 cm. from the target. Throughout irradiation the suspension was rapidly stirred and its temperature maintained between the limits of 6° C. and 12° C. by the circulation of ice-cooled water through the cooling coil. Samples were removed through a cotton-wool plugged side arm. Unirradiated suspensions retained their full viable counts throughout a period of 7 hours of rapid stirring with cooling in this chamber. 80 minutes' irradiation under our conditions reduced the viable count to *ca.* 2×10^4 organisms per ml.

(b) *By ultraviolet irradiation.*—Two ml. of suspension with 1×10^8 organisms per ml. was contained in a shallow glass vessel 3·5 cm. diameter located 50 cm. vertically below a Hanovia low pressure mercury discharge lamp delivering most of its radiation at a wavelength of 2657 Å. Exposure was conducted at room temperature for 2·5 minutes, by which time the viable count had been reduced to *ca.* 3×10^4 organisms per ml.

(c) *By nitrogen mustard.*—Tri-β-chlorethylamine hydrochloride (HN-3-HCl) was held as a 1 per cent (w/v) stock solution in distilled water. Of the suspension 1·98 ml. with 1×10^9 organisms per ml. in phosphate buffer pH 6·6 was incubated at 37° C. for 30 minutes. Of the stock solution of HN–3–HCl 0·02 ml. was then added and incubation continued for a further 30 minutes. This treatment reduced the viable count to *ca.* 5×10^6 organisms per ml.

3. *Enrichment of samples.*

(a) *By penicillin.*—Davis (1948, 1949*a*) obtained 80 per cent mutant cells among the survivors after subjecting irradiated suspensions of *E. coli* to a penicillin enrichment procedure. The enrichment depends on the selective bactericidal action of penicillin on growing cells, so that when a mixed population of mutant and non-mutant cells is incubated in minimal medium in the presence of penicillin, the non-mutant cells being capable of growth are eliminated, leaving the mutants unaffected. Lederberg and Zinder (1948) emphasized the necessity of modifying the technique to suit different organisms. In view of the number of possible variables in the technique it was not considered within the scope of this work to test all possible permutations and the technique finally adopted by us is possibly not optimal. We failed to obtain any mutants from X-irradiated samples subjected to the enrichment technique as described by Davis for *E. coli*. Experimentation with a mixed suspension consisting of equal numbers of Ty22 and of a mutant No. 479 (requiring a purine plus aneurin and isolated by the method of Beadle and Tatum) led to the development of a procedure giving a fifty-fold increase in the proportion of mutants to non-mutant cells among the survivors. However, when used on a mixture of 1000 Ty22 to 1 : 479 no mutants were recovered from 200 isolates. Applied to irradiated or mustard treated samples the procedure developed led to the isolation of a number of amino acid or vitamin-requiring mutants.

Samples after radiation or mustard treatment were grown overnight in complete medium. Of the complete medium culture 0·1 ml. was transferred to a complete medium slope, incubated 6 hours, emulsified and diluted in buffer and an inoculum of approximately one million organisms transferred to 4 ml. of

minimal medium from which the ammonium salts had been omitted. After 12 hours' incubation, ammonium salts to restore the concentration used in minimal medium, and penicillin to give a final concentration of 400 Oxford units per ml. were added (with the aid of an automatic device) and incubation continued for a further 6 hours. Penicillinase sufficient to inactivate residual penicillin was then added, the suspension diluted and plated on complete medium and the colonies, after 24 hours' incubation, examined for the presence of mutants. In many cases the suspension after penicillin treatment was again grown in complete medium and subjected to a second penicillin treatment, using the same procedure. Slight further enrichment was obtained by the double treatment. To avoid the repeated isolation of frequently occurring histidine or methionine requiring mutants these substances were added with the penicillin in later experiments.

A summary of the numbers of mutants obtained following penicillin enrichment procedures is given in Table I. Only those mutants having the same requirement derived from different samples are classified as independent mutants.

TABLE I.—*Mutants Obtained following Penicillin Enrichments.*

Mutagenic agent.	Type of enrichment.	Number of experiments.	Isolates tested.	Mutants obtained.	Independent mutants.
X-rays	Single	4	2316	18	13
	Double	4	2650	30	12
Ultraviolet	Single	8	3600	3	2
	Double	12	5366	61*	10
Nitrogen mustard	Single	4	1600	0	..
	Double	7	2768	6	5
	Totals	39	18,300	118	42

* Includes 52 mutants obtained from 200 isolates in one experiment. Of these 50 required histidine and 2 methionine.

(*b*) *By minimal supplementation.*—From the large number of cultures tested following treatment with penicillin no mutants requiring purines were obtained. Preliminary virulence tests with mutant 479 had shown these to be of interest and a method for their isolation, independent of penicillin treatment, was sought. Tests with known mixtures of Ty22 and the aneurin and purine-less mutant 479 showed that the colonies of each type could readily be distinguished when plated on minimal agar supplemented with limited amounts of purines sufficient to permit the mutants to develop into small colonies only. A method of detecting mutants by minimal supplementation was described by Davis (1949*b*). The medium employed by us was minimal agar plus 0·005 per cent (w/v) vitamin free casein hydrolysate and adenine, guanine, xanthine, hypoxanthine, cystosine and thymine each to 2·5 μM final concentration. Uracil was omitted, as it was found to be somewhat inhibiting. The constituents of minimal agar used in this method were of finest commercial grades, but not recrystallized.

Irradiated samples were grown overnight in complete medium supplemented with purines and pyrimidines, viable counts made and the appropriate dilution plated on supplemented minimal agar to give approximately 500 colonies per plate. After 48 hours' incubation all undersized colonies were isolated and tested

for mutants. The method was found to be simple and effective in eliminating a large proportion of non-exacting cultures prior to the final selection of mutants from mixed populations. It was only applied to X-irradiated samples. A summary of the numbers of mutants obtained in this way is given in Table II.

TABLE II.—*Mutants Obtained by Minimal Supplement Method following X-irradiation.* (*Dose rate* ca. 550 *roentgens per minute*).

Minutes irradiation.	Number of experiments.	Colonies examined.	Small colonies tested.	Mutants obtained.	Independent mutants.
0	1	5,800	12	0	0
40	1	12,000	31	5	5
60	8	98,600	212	39	23
80	7	125,800	301	43	23
100	5	22,850	68	3	3
Totals ..	21	259,250	612	90	54

4. *Selection of mutants.*

Initial attempts to isolate mutants from irradiated or mustard treated samples were made using the original method of Beadle and Tatum (1941), involving the transference of small inocula from complete medium agar to tubes of minimal medium and observing those which fail to grow on incubation. The method was very time-consuming and resulted in the selection of one mutant (No. 479) from 1755 cultures examined in this way. Two hundred and forty-one cultures tested subsequent to penicillin treatment yielded no mutants.

A considerable economy of time and materials was effected by adopting the following method, employed in the selection of all subsequent mutants. Penicillin treated samples were plated on complete medium agar to give 100 to 200 colonies per plate. Several hundreds of these taken at random were then transferred to correspondingly marked and numbered plates of minimal and complete medium, using the smallest inoculum possible just touched on the surface of the agar. Using a four-pointed platinum wire four colonies could be transferred to the pairs of plates between successive flamings. Two such wires used alternately permitted the transfer of 100 colonies to minimal and complete medium in 20 minutes. Incubation for 48 hours revealed non-exacting forms as robust colonies 2 to 3 mm. in diameter on the minimal agar whilst exacting forms remained as small film-like growths. The colonies on complete medium plates corresponding to the latter were then transferred to slopes for maintenance whilst their nutritional requirements were established. Small colonies isolated from minimal supplemented plates were selected for mutants in a similar manner. Fifty inocula were conveniently accommodated on each plate.

The exacting cultures obtained were confirmed as strains of *Bact. typhosum* firstly by their reaction to typhoid Type II E phage used in its critical test dilution for Ty22 and secondly by slide or tube agglutination with specific typhoid antisera.

5. *Determination of mutant requirements.*

An auxanographic method based on that described by Pontecorvo (1949) was used for the rapid determination of the growth factor requirements of mutants.

Plates of minimal agar were divided into a maximum of six sectors by 2 mm. wide channels, cut in the agar by means of a small " plough," and one well cut from the centre of each sector with a cork borer. Duplicate point inocula taken from mutant cultures on complete medium were placed radially around the wells, each of which contained a small volume of a different composite solution of growth factors. After 48 hours' incubation the solutions to which the mutants responded were in most cases obvious. The constituents of the active composite solutions were then tested individually or in combination by the same method to determine which were effective in promoting growth. All growth factor requirements determined by this plate method were confirmed by inoculation into tubes of minimal medium with and without the addition of the growth factors indicated.

Six different mutants could be conveniently spot-inoculated around each well, thus permitting the testing of six mutants against six different growth factors per plate. The channels were effective diffusion barriers preventing the added growth factors in one sector from masking the responses of inocula to those in adjacent sectors.

6. *Stability of mutants.*

A number of isolates failed to grow when first tested on minimal medium and were considered to be biochemical mutants. However, when subjected to the procedure used for the determination of mutant requirements they frequently showed full response to one factor, but evidenced instability by responding to a number of others on continued incubation. Such mutants, which presumably readily back-mutated to the non-exacting form, were considered unsuitable for further work and were discarded. Mutants responding to cystine or to glycine in the first instance were mostly of this very unstable type.

The quantitative responses of mutants to their respective growth factors added at various levels to tubes of minimal medium have been determined for most of those with different requirements to establish optimal levels for use in further work. On a number of occasions the instability of certain mutants was readily demonstrated during those determinations by the sudden recommencement of growth on prolonged incubation in those tubes containing insufficient growth factor addition to permit optimal growth of the mutant form.

Among the mutants retained by us those with requirements for cystine, glycine or proline appear particularly liable to reversion to the non-exacting condition. Similar reversion has been observed in those with arginine, phenylalanine and PAB deficiencies, whilst the remainder are apparently stable mutants or have low back mutation rates. Mutants with multiple deficiencies were found capable of loss of one or other or, more rarely, of all requirements, a property which made the establishment of the nutritional requirements of the original cultures a matter of some difficulty. In establishing the growth factor requirements of mutants the possibility of back mutation had to be constantly borne in mind and the growths obtained in tubes containing the supposed growth factors confirmed as those of mutants, and not of reverted organisms, by failure to grow on subculture into minimal medium.

7. *Types of mutants.*

Table III lists the different types of mutants obtained, their origin and the number of times independently isolated. As will be seen from Tables I and II,

the number of mutants isolated exceeds the number of independent mutants, since if more than one mutant with a particular requirement was isolated from any one sample, all such mutants were regarded as replicates derived from the original mutated cell by division and only one retained and included in Table III. Except where alternative requirements are indicated the mutants have not been observed to respond to the single addition of the other growth factors listed earlier.

DISCUSSION.

The very large increase in the proportion of mutant to non-mutant cells obtained by Davis (1949*a*) after penicillin treatment of irradiated suspensions

TABLE III.—*Types of Biochemical Mutants, How Obtained and Frequency of Independent Isolation.*

Growth factor requirements.	Number of independent isolations. Total.	By penicillin enrichment following treatment with: X-rays.	Ultraviolet.	HN_3.	By minimal supplement method after X-irradiation.
Glycine	5	2	1	..	2
Glycine or serine	1	..	..	..	1
Leucine	3	..	..	..	3
Valine + isoleucine	3	3	..	..	..
Proline	8	1	4	..	3
Cystine	10	3	..	..	7
Cystine or methionine	2	..	..	..	2
Methionine	16	5	3	..	8
Aspartic acid	3	1	1	1	..
Lysine	3	3	..	..	..
Arginine	5	2	..	..	3
Histidine	10	1	2	2	5
Phenylalanine	1	..	..	..	1
Tyrosine	1	..	..	..	1
Threonine	2	..	..	1	1
Aneurin	5	1	..	..	4
Nicotinamide	2	1	..	..	1
Pantothenate	1	1	..	..	..
Biotin	2	..	1	..	1
Para-amino benzoic acid	1	..	..	..	1
Any purine	1	..	..	..	1
Guanine or xanthine	1	..	..	..	1
Cytosine or uracil	3	..	..	..	3
Any purine + aneurin	3*	..	..	..	2
Valine + isoleucine + pantothenate	4	..	..	1	3
Not established	1	1	..	..	..
Totals	97	25	12	5	54

* Including mutant 479.

of *E. coli* was not achieved by us with *Bact. typhosum*. A pre-requisite for successful enrichment by this method is the establishment of conditions in a suspension in minimal medium in which mutant cells are theoretically unable to make a single division. In small scale experiments we have been able to demonstrate that Ty22 in the logarithmic phase of growth in minimal medium liberates into the medium sufficient growth factors to support considerable growth of all our different mutants with the exception of those requiring histidine, methionine, threonine, guanine, tyrosine or pantothenate. It is reasonable to assume, therefore, that in a mixed population of washed mutant and non-mutant cells syntrophic growth of many of the mutants could occur to a considerable extent, which, in the presence of penicillin, would lead to their elimination and reduce the efficiency of the enrichment. *E. coli* having a more perfectly balanced economy for growth in simple synthetic media, as evidenced by its higher growth rate than Ty22 in minimal medium, is probably less likely to support the development of mutants by growth factor over-production to such a large extent.

A number of interesting points arises from a survey of the types of mutants obtained. The high frequency with which mutants requiring histidine, methionine and cystine were selected is remarkable. Thus, of the 97 mutants obtained these three types account for over one-third, the remainder being composed of some 22 others. Had histidine and methionine requiring mutants not been specifically suppressed in the majority of penicillin enrichments their frequency would have been still higher. Several factors could contribute towards the higher frequency of isolation of particular mutants. Primarily this may result from a higher frequency of induction by virtue of the greater reactivity of particular genes with the mutagenic agents or their products, or, accepting the target theory for genetic mutation (Lea, 1947), from such genes occupying larger volumes so that they have a greater chance of the passage of a single ionizing particle through them for a given dose. The selection methods employed would favour those mutants having higher growth rates than the parent in complete medium, and those least able to grow syntrophically or more resistant to penicillin in enrichment treatments depending on its action.

It is most probable that among our mutants responding to cystine, to methionine or to histidine, blockage at different levels in the biosynthetic process of which these substances are terminal products are represented, and the frequency of a particular mutant may, to some extent, be indicative of the number of genetically controlled steps intervening between the presumed growth factor (e.g. histidine) and its nearest known precursor or precursors. With a fuller knowledge of the precursors involved in the synthesis of these three growth factors in *Bact. typhosum* and by a more comprehensive examination of the behaviour of our mutants to known precursors (e.g. sulphide in cystine synthesis) it is probable that these mutants could be assigned to a number of different types.

The relatively high frequency of mutants with a multiple requirement for valine plus isoleucine plus pantothenate is also notable. If biochemical mutation is a completely random process, other factors being equal it would be expected that multiple mutants would have requirements similar to those occurring with highest frequency among the simple mutants. However, no mutants requiring histidine plus methionine were obtained and no multiple mutants had a requirement for one or other of these amino acids plus another factor. Three valine plus isoleucine mutants were independently isolated and one pantothenate as

opposed to the four multiple mutants requiring all three substances. It is probable that the requirement for valine plus isoleucine is a similar phenomenon to that described by Bonner (1946) for *E. coli* mutants, where the blockage of a single gene controlled conversion of the keto acid analogue of isoleucine results in inhibition of valine synthesis. These multiple mutants have been observed to revert to the loss of a requirement for pantothenate or for valine plus isoleucine suggesting that two genes are involved in the mutation rather than one gene controlling the synthesis of two different substances. The three independent isolations of purine plus aneurin-requiring mutants is similarly notable.

The employment of the minimal supplement method effected a considerable economy of time and labour in the selection of biochemical mutants and yielded a variety of different types. The method was adopted mainly to obtain purine and pyrimidine mutants and the casein hydrolysate added primarily to induce more rapid growth. Supplementation with specific amino acids at known concentrations rather than with casein hydrolysate would probably assist the recovery of mutants requiring these substances as growth factors. However, with a casein hydrolysate addition, one in seven of the small colonies isolated was found to be exacting, whereas following penicillin enrichment an average of 155 were tested for each mutant obtained. Supplementation of complete medium with additional purines and pyrimidines possibly assisted the recovery of mutants with these requirements by the minimal supplement method, although all were found to grow abundantly on unsupplemented complete medium. Since no specific vitamin additions were made to minimal supplement plates it is presumed that the vitamin-requiring mutants isolated developed syntrophically or from vitamins present as impurities.

The mutants obtained provided a variety of material for the investigation of the effects of biochemical mutation on the virulence of *Bact. typhosum*, which will be reported in a subsequent paper.

SUMMARY.

A number of biochemical mutants of *Bact. typhosum* with requirements for amino acids, vitamins, purines or pyrimidines have been obtained following treatment with X-rays, ultraviolet light or nitrogen mustard, as a preliminary to a study of the effects of biochemical mutation on virulence.

The use of point inocula and solid media was found to facilitate the selection of biochemical mutants from mixed population and the determination of their growth factor requirements.

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THE EFFECTS OF BIOCHEMICAL MUTATION ON THE VIRULENCE OF *BACTERIUM TYPHOSUM*: THE VIRULENCE OF MUTANTS.

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A PREVIOUS paper (Bacon, Burrows and Yates, 1950) described the induction, selection and characterization of a number of biochemical mutants of *Bact. typhosum* derived primarily for the purpose of studying the effects of biochemical mutation on virulence. Some general aspects of the complex problem of virulence in bacteria have been included in a recent review by Watson and Brandley (1949). The absence of a convenient test animal readily susceptible to infection by small numbers of typhoid organisms has been a considerable handicap to the progress of experimentation on typhoid. Nevertheless the method developed by Felix (1938), using organisms suspended in Ringer's solution and injected intraperitoneally into mice, has proved useful in differentiating strains of typhoid with respect to virulence, despite the fact that relatively large numbers of the most virulent organisms are required to induce a fatal intection in this animal.

It is, however, possible with the aid of hog gastric mucin to reduce the numbers of typhoid organisms capable of initiating the fatal infection of mice to a very low figure (Review: Olitski, 1948). Batson, Landy and Brown (1950) in a recent comparison of the efficiencies of methods employing intraperitoneal injection into mice of organisms suspended in saline or in mucin, or the injection of saline suspensions intracerebrally, concluded that, of the three methods, greatest differentiation of typhoid strains with respect to virulence was obtained by that employing mucin.

Although of value in differentiating strains of lower virulence from one of high virulence, the very small numbers of organisms comprising an average lethal dose of the latter suspended in mucin precludes the use of this adjuvant for the detection of strains showing increased virulence over that of a highly virulent strain taken as a standard. The parent strain Ty22 from which our mutants were derived is of a high virulence, and as it was considered possible that some mutants may show enhanced virulence, the use of mucin was avoided in this work.

Most workers are in agreement that strains of *Bact. typhosum* showing the highest virulence for mice are culturally smooth, relatively opaque and possessing maximum amounts of both Vi and O somatic antigens, whilst rough strains or those devoid of either Vi or O show considerably lowered virulence. To determine whether biochemical mutation materially affected the production of these two antigens considered essential for maximum virulence, the agglutination of our mutants by typhoid Vi and O antisera was examined concurrently with tests of virulence.

MATERIALS AND METHODS.

Media.

Tryptic digest meat broth (TMB) or agar (TMA) were employed as complete medium. Glucose-ammonium-phosphate medium prepared as previously described (Bacon *et al.*, 1950) was used as minimal medium. Phosphate buffer contained 4·5 g. KH_2PO_4 per litre and was adjusted to pH 7·4 with NaOH.

Strains.

The strains used were biochemical mutants derived from a single ammonia assimilating strain Ty22 subjected to X-rays, ultra-violet irradiation or to nitrogen mustard. In all some 93 such mutants representing 25 different types have been examined. They are listed together with their growth factor requirements in Table II. Cultures were maintained in the dry state by the gelatin-ascorbic acid method described by Stamp (1947) and stored *in vacuo* at $\pm$ 2° C.

Suspensions.

A standard procedure was adopted for the preparation of all suspensions used for virulence experiments. Dried pellets were emulsified in TMB, plated on TMA to give an area of confluent growth and a number of separate colonies, and incubated 16 hr. at 37° C. The plates were examined for purity, refrigerated 7 hr., a representative inoculum from the edge of the confluent growth seeded to minimal agar supplemented with optimal amounts of the necessary growth factor(s), and incubated 18 hr. at 37° C. in *ca.* 5 per cent CO_2 95 per cent air. After cooling in the refrigerator for 30 min. the growths were emulsified in ice cold buffer to the opacity required for injection, viable counts made on these suspensions by the method of Miles, Misra and Irwin (1938), and the suspensions injected into mice with the minimum of delay.

Agglutinating suspensions were similarly prepared, using normal saline at room temperature in place of cold buffer.

Virulence tests.

White mice (Hough strain) equally divided as to sex and between weight limits of 18 to 22 g. were used throughout. They were received in batches of 200 to 400 and assigned to separate containers the day before injection. The required dose contained in 0·5 ml. was injected intraperitoneally and the animals observed over a seven-day period. Two scales of testing were used :

(a) *Minor tests.*—All mutants were subjected to a minor virulence test in which 50 million cells of each mutant, estimated on an opacity basis, were injected into each of 10 mice. Six to 18 mutants, buffer and Ty22 controls were assayed per experiment, and those mutants apparently altered in virulence on the evidence of such tests were selected for the major tests. Limitation in the availability of test animals made larger scale testing of all mutants impracticable within a reasonable period. Major tests were therefore limited to those mutants appearing altered in virulence in the minor tests and to one representative of each different type.

(b) *Major tests.*—Eighty mice were used for each mutant. One to three mutants were assayed against Ty22 per experiment, using four graded doses, estimated from smaller scale tests to cover the A.L.D. of each culture, each injected

into 20 mice. Probit mortality in 7 days–log dose regression lines were constructed and the relative virulence of the mutants with respect to Ty22 determined by the methods of probit analysis. Experiments in which more than one of the 20 buffer control mice died were repeated. Of 580 control mice used throughout these tests no deaths were recorded within the first 5 days of the experiments, and a total of only 4 within the seven-day periods. Deaths among control mice occurred only in experiments conducted between January and March of the 11 months' duration of these experiments.

Agglutination tests.

Living agglutinating suspensions were standardized to 2300 million organisms per ml. (opacity 5 of Burroughs Wellcome scale), and one drop added to 1 ml. of the Vi or O serum dilutions in 5 cm. × 1·2 cm. tubes. The tubes were incubated 2 hr. at 37° C. in an incubator and read after standing overnight at room temperature. Suspensions killed by heating 30 min. in a boiling water bath were similarly examined for their O-agglutinability.

RESULTS.

General characters of mutants.

The majority of mutants were colonially indistinguishable from the parent Ty22. They grew readily on TMA, producing circular, smooth, more or less opaque brownish colonies. The exceptions noted were mutants 9749 and 20085 (histidine requiring) giving less smooth colonies, and mutant 20092 (guanine or xanthine) giving normal smooth colonies about one-half the diameter of those of Ty22. Aspartic-less mutants gave colonies with a distinctive annular depression.

Similarly the majority of mutants were morphologically indistinguishable from the parent. The variations noted among those dissimilar were 9749, in which the cells were shorter and aggregated into streptococcus-like chains, 20085 with many long unsegmented filamentous forms, 20037 (methionine) a plump coccobacillus, 20058 (cystine) and 20092 both occurring as plump coccobacilli, many in short chains.

Phage sensitivity.

Without exception the mutants were fully sensitive to lysis by Type IIE typhoid Vi phage used in its critical test dilution for Ty22, when tested by the plate method described by Craigie and Yen (1937).

Virulence of mutants.

Table I is illustrative of minor tests of virulence in which mutant cultures were injected into 10 mice, each receiving a dose of 50 million organisms estimated on an opacity basis. Although all injected suspensions were standardized to the same opacity, their viable counts varied more than could be attributed to limitations in the accuracy of opacity determination. This discrepancy between the expected and apparent viable count made the interpretation of virulence tests a matter of some difficulty. The question that arose was whether similar doses of different organisms were better represented by equal volumes of suspension of equal opacity or by equal numbers of viable organisms. Presumably it is the mass of living bacterial matter injected rather than the numbers of individual

or groups of organisms into which it is divided that is of primary importance in a suspension used in a test of virulence. Measurements of opacity would take into account variation in the morphology of the organisms in suspension without differentiating between living and dead or between organisms of differing transparency, whereas viable counts make no allowance for differences of morphology, and can only be reliable when all cells are distinct individuals. Thus neither method of estimating the dose of living bacterial matter administered is fully reliable when comparing the virulence of suspensions of different organisms which may differ morphologically or in the transparency of individual cells.

As it is improbable that the opacity measurements would be greatly in error from the presence of dead cells in physiologically young cultures, whilst viable counts are considerably affected by aggregation or variation in the size of cells, we consider the relative virulence assessed on opacity dosages is the more reliable. Assessments were made on both bases, but only those on an opacity basis are recorded in the tables.

In the majority of tests Ty22 injected at a level of 50 million organisms (opacity) resulted in 8 out of 10 fatal infections. This number varied on occasions between 4 and 10 out of 10. The variation noted may have been the result of normal sampling fluctuations, of change of virulence of Ty22 or of change in resistance to infection of the mice. On occasions when Ty22 killed a low or high proportion of the mice, those mutants which we have reason to believe are not significantly altered in virulence tested in the same experiments also killed a low or high proportion, suggesting a general higher or lower resistance of the animals to infection rather than a change in virulence of the standard Ty22. The two experiments recorded in Table I illustrate this point.

From these two experiments mutants 20063, 20025 and 20085 were regarded as altered in virulence and subjected to major tests. Mutant 20071 was reassayed in a subsequent minor test.

TABLE I.—*Illustrating Minor Tests of Virulence.*

Expt. number.	Culture.	Growth factor.	Dose organisms × 10^{-6}. On opacity measurement.	Dose organisms × 10^{-6}. On viable count.	Number of mice injected.	Deaths in— 3 days.	Deaths in— 7 days.
31	(buffer)	..	—	—	10	0	0
	Ty22	—	50	69	,,	4	4
	20063	PAB	,,	49	,,	0	0
	20022	Phenylalanine	,,	67	,,	5	5
	20045	Threonine	,,	56	,,	6	6
	20068	Cytosine	,,	42	,,	6	6
	20025	Leucine	,,	47	,,	1	1
	20075	Glycine	,,	65	,,	6	6
37	(buffer)	..	—	—	10	0	0
	Ty22	—	50	53	,,	8	8
	20074	Aneurin	,,	53	,,	9	9
	20105	,,	,,	47	,,	8	8
	20110	Histidine	,,	54	,,	9	9
	20085	,,	,,	13	,,	0	0
	20073	,,	,,	50	,,	8	8
	20087	Methionine	,,	59	,,	8	8
	20084	Proline	,,	54	,,	9	9
	20071	Cystine or methionine	,,	60	,,	10	10

TABLE II.—*List of Mutants with their Growth Factor Requirements and Numbers of Mice Killed out of 10 by Doses of 50 Million Organisms.*

Growth factor.	Mutant.	Deaths. Mutant.	Deaths. Ty22.
Methionine	3306	7	8
	4438	8	8
	5459	8	8
	7010	7	8
	20012	5	8
	20015	9	8
	20018	7	7
	20023	8	10
	20031	8	10
	20037	9	10
	20056	9	10
	20072	9	10
	20083	5	7
	20087	8	8
	20114	9	10
Cystine	2651	8	9
	6569	8	9
	6721	8	9
	20021	8	8
	20040	4	4
	20058	0	10
	20059	6	6
	20120	6	4
	20125	8	4
Cystine or methionine	20062	9	10
	20071	6	6
Arginine	6775	9	9
	7226	5	9
	20028	6	6
	20046	8	4
	20069	9	10
Leucine	20025	1	8
	20082	1	9
	20113	4	9
Histidine	3160	5	8
	3663	3	8
	5555	4	8
	6346	7	8
	9749	0	8
	20030	8	10
	20065	4	10
	20073	8	8
	20085	0	8
	20110	9	8
Proline	9468	9	9
	11392	6	8
	13302	9	10
	16891	9	8
	20011	5	4
	20067	2	4
	20084	9	8
	21857	9	8
Glycine	7072	7	9
	10833	8	10
	20034	9	10
	20075	6	4
Glycine or serine	20066	9	10
Lysine	3234	7	9
	7330	9	9
	12190	8	10
Valine + isoleucine	7168	8	10
	9355	9	8
	12559	4	8
Threonine	20045	6	4
	21403	8	8
Aspartic acid	8505	0	8
	19341	0	8
	19662	0	8
Phenylalanine	20022	5	4
Tyrosine	20100	7	7
Valine + isoleucine + pantothenate	16265	9	8
	20026	2	6
	20078	6	6
	20096	4	4
Pantothenate	10020	9	10
Aneurin	20002	6	8
	20032	9	7
	20039	4	6
	20074	9	8
	20105	8	8
Biotin	13461	5	8
	20053	7	7
Nicotinamide	8974	9	10
	20035	6	7
PAB	20063	0	4
Cytosine	20057	8	7
	20068	6	4
	20112	7	8
Purine	20080	0	8
Guanine or xanthine	20092	0	8
Purine + aneurin	479	0	8
	20095	0	6
	20102	0	8
?	20001	4	8

Table II summarizes the results of minor virulence tests of all our mutants, giving the numbers of mice killed by the mutants in comparison with the numbers killed by an equal (opacity) dose of Ty22 tested in the same experiment.

An illustrative major virulence test is presented in Table III. Tables IV and V summarize the results of all such tests.

TABLE III.—*Illustrating Major Tests of Virulence.*

Culture.	Growth factor.	Dose organism × 10^{-6}. Opacity.	Viable.	Mice injected.	Deaths in— 3 days.	7 days.	Relative virulence with limits (P = 0·05).
Ty22	..	10	7·0	20	7	8	1·0
		20	14·0	„	11	12	
		40	28·0	„	13	13	
		80	56·0	„	17	17	
20040	Cystine	10	10·0	„	5	5	0·72
		20	20·0	„	6	6	
		40	40·0	„	14	14	(0·43–1·1)
		80	80·0	„	17	17	
20084	Proline	10	9	„	3	3	
		20	18	„	9	9	0·64
		40	36	„	12	13	(0·38–1·0)
		80	72	„	15	15	
20080	Purine	1000	600	„	1	3	0·005
		2000	1200	„	5	6	(0·003–0·009)
		4000	2400	„	11	11	
		8000	4800	„	17	17	

TABLE IV.—*Virulence of Representative Mutants of Each Growth Factor Type.*

Growth factor(s).	Mutant.	Relative virulence with limits (P = 0·05).
Methionine	5459	0·8 0·5 –1·2
Cystine	20040	0·7 0·4 –1·1
Cystine or methionine	20071	1·3 0·8 –2·2
Arginine	6775	1·2 0·7 –1·7
Leucine	20025	0·4 0·2 –0·6
Histidine	6346	0·5 0·3 –0·8
Proline	20084	0·6 0·4 –1·0
Glycine	20034	0·4 0·3 –0·7
Glycine or serine	20066	0·6 0·4 –0·9
Lysine	3234	1·4 0·8 –2·5
Valine + isoleucine	9355	0·6 0·3 –1·2
Threonine	20045	1·2 0·7 –2·0
Aspartic acid	8505	0·1 0·06–0·2
Phenylalanine	20022	1·4 0·7 –2·7
Tyrosine	20100	1·1 0·7 –2·0
Valine + isoleucine + pantothenate	16265	0·7 0·5 –1·1
Pantothenate	10020	1·3 0·9 –2·6
Aneurin	20039	0·7 0·3 –1·3
Biotin	20053	0·5 0·3 –1·2
Nicotinamide	8974	1·2 0·6 –2·2
PAB	20063	0·1 0·08–0·2
Cytosine	20057	0·8 0·5 –1·2
Purine	20080	0·005 0·003–0·009
Guanine or xanthine	20092	0·03 0·02–0·06
Purine + aneurin	479	0·03 0·01–0·04

TABLE V.—*Virulence of Mutants Estimated from Minor Tests to have Altered in Virulence.*

Mutant.	Growth factor(s).	Relative virulence with limits (P = 0·05).	
9749	Histidine	0·05	0·03 –0·07
20085	,,	0·2	0·1 –0·3
20058	Cystine	0·02	0·01 –0·03
8505	Aspartic acid	0·1	0·06 –0·2
19341	,, ,,	0·1	0·06 –0·2
19662	,, ,,	0·1	0·09 –0·2
20063	PAB	0·1	0·08 –0·2
20080	Purine	0·005	0·003–0·009
20092	Guanine or xanthine	0·03	0·02 –0·06
479	Purine + aneurin	0·03	0·01 –0·04
20095	,, ,,	0·01	0·004–0·02
20102	,, ,,	0·03	0·02 –0·05

In vivo *reversion of mutants.*

In view of the ease with which certain mutants were known to lose their growth factor requirements in *in vitro* studies it was important to consider their stability *in vivo*. In earlier experiments organisms were re-isolated on TMA from the spleens of animals surviving the injected dose of mutant cultures on the seventh day after injection, and tested for their dependence on added growth factors for development on minimal medium. Typhoid organisms were invariably recoverable from the spleens after this time. Twenty single colonies taken at random from the TMA-plates were spot inoculated to minimal medium plates with and without added growth factor(s). After two days' incubation, growth of all inocula on supplemented minimal medium but of none on unsupplemented medium was indicative of no appreciable reversion to the non-exacting condition. Isolations from 34 mice injected with different mutant cultures were all found to have retained their growth factor requirement.

It was, however, of greater importance to observe the proportion of reverted organisms in mice succumbing to infection rather than in surviving animals. Accordingly, in subsequent experiments, animals on the point of death on the first or second days following injection were killed by cervical dislocation, a loopful of peritoneal fluid emulsified in minimal medium, serially diluted and plated on growth factor supplemented and unsupplemented minimal medium. A comparison of the numbers of colonies developing on the two media allowed a good estimate to be made of the maximum ratio of reverted to mutant organisms. Isolations made in this manner from moribund mice almost invariably yielded pure cultures of typhoid organisms. Similar isolations from dead animals were frequently heavily contaminated with coliform bacteria. Of 59 isolations from moribund mice injected with 33 different mutant cultures, 4 showed *ca.* 100 per cent reversion (mutants 9749 and 20058), 1 showed 9 per cent (20092), 5 between 1 per cent and 0·01 per cent, and the remaining 49 isolations less than 0·01 per cent reversion to the non-exacting form. The low incidence of reversion observed suggests that the majority of our assessments of the relative virulence of mutants with respect to Ty22 is unlikely to be subject to any great error from *in vivo* instability of mutants.

TABLE VI.—*The Serological Behaviour of Selected Mutants of High and Low Virulence.*

	Culture.	Growth factor.	Agglutination.																									
			Living suspensions.														Heat-killed suspensions.											
			Vi serum dilution.						O serum dilution.								O serum dilution.											
			100	200	400	800	1,600	3,200	6,400	250	500	1,000	2,000	4,000	8,000	16,000	32,000	64,000	250	500	1,000	2,000	4,000	8,000	16,000	32,000
Mutants of high virulence	Ty22	—	6	6	6	6	4	1	—	2	—	—	—	—	—	—	—	—	6	5	5	4	4	3	1	—
	20087	Methionine	6	6	6	6	5	1	—	4	4	1	1	—	—	—	—	—	6	6	5	5	3	1	—	—
	20032	Aneurin	6	6	6	6	5	2	—	5	4	3	1	—	—	—	—	—	6	5	5	4	2	1	1	—
	8974	Nicotinamide	5	5	5	5	4	1	—	—	—	—	—	—	—	—	—	—	6	6	5	3	2	—	—	—
	20057	Cytosine	6	5	5	3	3	3	1	5	5	4	2	1	—	—	—	—	6	6	5	4	2	1	1	—
	20084	Proline	6	6	6	5	5	2	—	5	5	2	1	—	—	—	—	—	6	6	6	4	2	1	—	—
	20100	Tyrosine	6	4	4	4	3	1	—	5	3	2	1	—	—	—	—	—	6	6	6	5	2	1	—	—
	10020	Pantothenate	5	5	5	5	3	1	—	1	—	—	—	—	—	—	—	—	6	6	5	3	2	—	—	—
Mutants of low virulence	20063	PAB	5	5	4	3	2	1	—	1	1	1	—	—	—	—	—	—	6	6	4	3	2	1	1	—
	8505	Aspartic	6	6	6	6	5	2	—	3	1	—	—	—	—	—	—	—	6	5	5	4	4	3	3	—
	19321	,,	5	5	5	5	3	2	—	4	4	2	2	—	—	—	—	—	6	6	4	3	2	—	—	—
	20080	Purine	6	6	5	3	3	1	—	5	1	—	—	—	—	—	—	—	6	5	5	3	2	1	—	—
	20092	Guanine	6	5	5	3	1	—	—	2	1	—	—	—	—	—	—	—	6	6	5	4	2	1	—	—
	479	Purine + aneurin	6	6	6	6	5	2	—	3	2	1	—	—	—	—	—	—	6	5	5	4	3	3	3	—
	20102	,, ,,	6	4	4	4	3	1	—	5	5	2	1	—	—	—	—	—	6	6	6	5	3	1	—	—
Standard : Vi	T6S	—	6	6	5	5	2	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
O	0901	—	2	1	—	—	—	—	—	6	6	6	5	3	2	1	—	—	6	6	6	4	2	1	—	—

Degrees of agglutination : 6 = Agglutination complete, supernatant clear ; 5 = agglutination almost complete, supernatant turbid ; 4 = agglutination just visible with naked eye ; 3 = agglutination easily visible with lens ; 2 = trace visible with lens ; 1 = faintest trace visible with lens.

Agglutination of mutants.

With the exception of those strains referred to below, all mutants, on the evidence of the agglutinability of living suspensions by a Vi antiserum and of heat-killed suspensions by an O antiserum, were found to be of the Vi + O type. Living suspensions resisted O-agglutination in varying degrees. The O-resistance of naturally occurring Vi + O strains is commonly regarded as an index of their virulence for mice, the less O-agglutinable strains being the more virulent. However, with our artificially selected biochemical mutants the behaviour of living suspensions towards our O-antiserum was not always indicative of their relative virulence. A selection of a number of virulent and relatively avirulent mutants with their serological behaviour is presented in Table VI to illustrate the difficulty of differentiating the two types from serological evidence alone.

Three strains differed in their agglutination responses from the majority. Strains 9749 and 20085 produced slightly unstable suspensions in 0·85 per cent saline from growths on supplemented minimal medium. Grown on TMA and suspended in 0·1 per cent saline both were sufficiently stable to indicate strain 9749 to be of the Vi + O type, but less rich in these antigens than Ty22, and 20085 to be markedly deficient in O antigen. Mutant 20058 agglutinated in our Vi antiserum to a distinctly lower titre than all other mutants. These three mutants are altered morphologically and of lowered virulence.

DISCUSSION.

At the outset of this work it was visualized that biochemical mutation may possibly result in an enhancement of the virulence of our organism. The accumulation of synthetic products whose further metabolism is prevented by mutational blockage is well known in studies of biochemical mutants in fungi and bacteria. Such intermediate metabolites may well be structurally dissimilar from those of the host, resulting in the blockage of important synthetic mechanisms in a competitive manner and facilitating the establishment of a fatal infection by the mutant. Secondly, it is conceivable that an organism with a particular growth factor requirement would, on injection into a host, compete with it for that factor. Further, by nature of its larger surface area/volume ratio the organism could conceivably absorb this factor from the body fluids more efficiently than the host cells, resulting in the starvation of the latter with respect to the factor. If this were normally in limited supply and vital for the correct functioning of the cells, such starvation would probably lead to a lowering of resistance to infection and be reflected in an apparent increase in virulence of the parasite, providing that the mutation had not simultaneously decreased the resistance of the organism to the normal anti-bacterial mechanisms of the host.

Our results concerning the virulence of biochemical mutants representing a number of different growth factor types do not lend support to these views as applied to the infection of the mouse with *Bact. typhosum* mutants. However, it is possibly the case that growth factor dependence may well enhance the virulence of an organism initiating a less acute disease, by creating zones of growth factor deficiency in the microenvironment of organisms lodged in the host, rather than of one initiating a rapidly fatal toxaemia, such as follows the injection of large numbers of typhoid organisms into the mouse.

Probit analysis revealed no significant divergence from parallelism or any

evidence of heterogeneity between the regression lines of parent and mutants throughout the entire range of virulence encountered, suggesting the mutants to exert a similar pharmacological effect to that of Ty 22.

Surveying the results of major virulence tests with the supporting evidence of minor tests, it is evident that the majority of our mutants have not significantly changed in virulence. Of amino-acid requiring mutants slight lowering of virulence is associated with dependence on added leucine, histidine or glycine. An aspartic acid requirement, however, is seen to result in 3 out of 3 instances in a marked decrease in virulence. Two out of 10 histidine and 1 of 11 cystineless mutants (9749, 20085 and 20058) differ from the other mutants with these requirements in being of considerably lower virulence than the parent. Loss of virulence here may be a direct result of biochemical mutation affecting a particular step in the synthesis of histidine or cystine, or alternatively may arise from a simultaneous "non-biochemical" mutation, leading to the antigenic, colonial or morphological variations previously described.

Among 15 mutants with requirements for vitamins of the B group, loss of virulence is confined to the one mutant unable to synthesize para-amino-benzoic acid. Here loss of virulence is not associated with any morphological change or demonstrated major antigenic differences from the parent.

The most notable loss of virulence is associated with those mutants deficient in their ability to synthesize purines. Five independently isolated mutants of this type have a relative virulence of the order of 0·005 to 0·03 of that of the non-exacting strain, so that an average lethal dose is in the region of 2000 million organisms. The relatively insignificant effect on virulence occasioned by mutation to dependence on added aneurin, shown by five such independently isolated mutants, would suggest the mutation to purine dependence is the major factor resulting in loss of virulence in those mutants having a double requirement for a purine plus aneurin.

Biochemical mutation can be visualized to lead to a loss of virulence in a number of ways. A dependence on an exogeneously supplied growth factor may limit the production of non-vital constituents of the bacterial cells, which, however, are of importance from the aspect of virulence, if such constituents require the participation of the growth factor in their synthesis. It is possible that non-vital surface complexes, important for the manifestation of virulence, are produced from excess syntheses of the vital requirements of the virulent organism. Should the growth factor of a particular type of mutant be one moiety of such a complex, in its absence due to limitation in supply or to the inability of the mutant to incorporate exogeneously supplied growth factor, such a complex may not be produced or may be formed in a non-functional state. The complete absence or the limited availability of the required growth factor in the host preventing the multiplication of the injected mutant to a toxic level presents a third possible mechanism whereby biochemical mutation could lead to a loss of virulence of the mutant.

We have in Ty22 and our mutants of low virulence an opportunity to study mouse virulent and avirulent strains of *Bact. typhosum*, which in all probability differ from each other in the presence or absence of single genes concerned with the synthesis of specific growth factors. The great loss of virulence shown by purine requiring mutants is of particular interest in that these mutants conform in colonial character, antigenic structure and Vi phage sensitivity to the accepted

criteria defining virulent strains of typhoid. Studies with our avirulent mutants, with the object of determining reasons for biochemical mutation resulting in loss of virulence, are in progress.

SUMMARY.

1. The effect of biochemical mutation on the virulence of *Bact. typhosum* was investigated, using 93 mutants representing 25 different growth factor types.

2. The majority of mutants retained full virulence.

3. Slight loss of virulence was associated with a requirement for leucine, histidine or for glycine.

4. Three independently isolated aspartic acid and one para-amino-benzoic acid mutant were reduced in virulence to *ca.* 0·1 of that of the parent.

5. Five independently isolated purine-requiring mutants were reduced in virulence to *ca.* 0·02 of that of the parent.

6. The majority of both virulent and avirulent strains was unaltered in colony form, morphology or major antigenic structure.

7. Three anomalous strains of low virulence had morphological or other variation in addition to a growth factor requirement.

8. All mutants retained sensitivity to typhoid Vi phage.

9. The O-agglutinability of mutants was not infallibly indicative of their mouse virulence.

10. Possible mechanisms leading to alteration of virulence by biochemical mutation are discussed.

We gratefully acknowledge the advice and criticism offered by Dr. D. W. Henderson throughout this work. Our thanks are expressed to Mr. S. Peto for statistical interpretation of our data, to Miss B. Alkins for excellent laboratory assistance, and to the Director of the Public Health Laboratory, Colindale, for gifts of antisera.

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INFLUENZA VIRUS HAEMAGGLUTINATION. THE MECHANISM OF THE FRANCIS PHENOMENON.

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THE phenomenon described by Francis (1947) served to focus attention on serum constituents, other than specific antibodies, which can inhibit influenza virus haemagglutination, more especially if the virus has been degraded by heat treatment. Susceptibility of Francis inhibitor to the destructive action of the virus enzyme, which attacks red blood cell receptors, seemed to offer an adequate explanation of the phenomenon (Hirst, 1948*b*), and more recently Stone (1949) has demonstrated in a number of strains of virus a close correlation between their susceptibility to inhibition by Francis inhibitor and the degree of inactivation of their enzymes. According to this theory, living virus is able to free itself from, and destroy, attached inhibitor, while heated virus, being without enzymic activity, is unable to do so with the result that the haemagglutinin receptors remain blocked.

This explanation is, however, untenable, since the phenomenon can be as readily demonstrated at 0° C. as at room temperature (Anderson, Burnet, Fazekas de St.Groth, McCrea and Stone, 1948; Stone, 1949). Nevertheless, it is still widely accepted, and used as a basis for further hypotheses relating to virus infectivity. In this laboratory we have recently produced by ether treatment a virus preparation which, while retaining its full enzymic activity as measured by its capacity to elute from red blood cells, is as efficient an indicator of Francis inhibitor as its heat-treated counterpart. There are numerous minor observations which conflict with the enzyme hypothesis, but these two in themselves appear to us to be conclusive.

An alternative hypothesis has been advanced by Burnet and his co-workers, that there exists a dynamic equilibrium in the competition between inhibitor and red cell for the virus receptors, the degree of inhibition reflecting the relative avidities of the virus particle for the two competing substrates (Anderson *et al.*, 1948). Burnet suggests that heat treatment so modifies the virus surface as to increase its affinity for inhibitor at the expense of the red cell.

The importance of understanding the mechanism of inhibitor action is obvious and it seemed to us that Burnet's hypothesis could be put to experimental test. It will be shown that it, likewise, is untenable, and an explanation based on the experimental findings will be presented.

* Seconded for virus research from the staff of the Public Health Laboratory Service.

Definitions.

So much confusion exists in the literature of virus haemagglutination that the precise meaning which we intend to attach to certain terms must be made clear.

The Francis phenomenon or Francis effect was originally described in relation to Influenza B (Lee) (Frances, 1947), and a number of sera, both " normal " and immune. Exactly the same phenomenon occurs with strains of Influenza A, namely, an increase in the inhibitory action of many normal sera against a standard agglutinating dose of virus when the virus is heated, usually at 56° C. for 30 minutes. We use the terms, therefore, in respect of Type A virus and the sera of normal rabbits.

The terms " Francis inhibitor " and " non-specific inhibitor " have been the cause of much confusion. By virtue of its original connection with Type B virus, Francis inhibitor is sometimes used to denote inhibitor to B virus only. Sometimes it covers inhibitors to both A and B viruses, and again it is used to denote inhibitor to heated as opposed to living virus, the term " non-specific inhibitor " being used to denote inhibitor to the latter. We, therefore, intend to avoid use of these terms, and will use instead the term " serum inhibitor " or simply " inhibitor," without prejudice to the question as to whether inhibitors to A and B strains are qualitatively distinct.

The question of whether inhibitor to heated virus is qualitatively different from that effective against living virus is fundamental to the mechanism of the Francis response and requires more detailed consideration. Briody (1950) gives a concise review of the present position. Two qualitatively different inhibitors are held to be involved. The inhibitor of living virus, called non-specific inhibitor, acts on living A and B viruses; the inhibitor of heated virus called Francis inhibitor acts on heated A and B viruses. The demonstration of these two inhibitors, as qualitatively distinct entities is attributed to McCrea (1946, 1948) and to Burnet and McCrea (1946). The main distinction claimed to exist between the two is briefly as follows: Non-specific inhibitor of living virus is a γ-globulin, completely recoverable at 33 per cent saturation with ammonium sulphate, rapidly destroyed at 62° C. and relatively unaffected by periodate. Francis inhibitor of heated virus, on the other hand, is a mucoprotein, not completely recovered at 33 per cent saturation with ammonium sulphate, stable to 100° C. and rapidly inactivated both by periodate and the receptor destroying enzyme (R.D.E.) of *V. cholerae.*

In contradistinction to these findings Hirst (1948*a*, 1948*b*) found that non-specific inhibitor was stable to 100° C. for 15 minutes, while at 62° C. a complex reaction took place, probably involving other serum constituents. Furthermore it was destroyed by periodate and by living virus. Van der Veen and Mulder (1950) found that non-specific inhibitor was absorbed out by heated Lee B virus, was destroyed by incubation with living Lee B virus, and was destroyed by periodate and R.D.E.

A partial explanation of these contradictions may lie in the fact that McCrea's work on non-specific inhibitor was done in 1946 before the Francis phenomenon had been described, and there is no record that purified non-specific inhibitor was ever tested against heated virus. In his work on Francis inhibitor in 1948, a living A virus (BELD) and a heated B (Lee B) were used to measure non-specific and Francis inhibitor activity respectively. Living B and heated A were not used.

Thus the comparison made was not between inhibitors to living and heated virus, but between inhibitors to living A and heated B. At the same time it is stated that "even purified mucoid . . . may still show moderate inhibitory activity against haemagglutination by some active viruses" (McCrea, 1948). Moreover, since the majority of Francis inhibitor activity was recovered at 33 per cent saturation with ammonium sulphate, it follows that the fraction tested as non-specific inhibitor must also have contained the majority of the Francis inhibitor activity. There is thus no evidence that the purified mucoid is not a portion broken off, by the drastic technique of its preparation, from a larger molecule incorporating both activities.

To summarize, we do not feel that the evidence is convincing that the activities against living and heated virus are represented by qualitatively distinct inhibitors. It seems probable that McCrea's concept of a protein-mucoprotein complex is nearer the truth. The work reported in this paper has been restricted to the serum of normal rabbits and a P.R.8 strain of Influenza A virus. By "serum inhibitor" we, therefore, imply a substance or complex present in the serum of normal rabbits which is capable of inhibiting the haemagglutinating activity of the P.R.8 strain used. To what extent generalizations can be made from the mechanism of the interaction studied will be discussed in a later section.

MATERIALS AND METHODS.

Virus.

The virus used in these investigations designated P.R.8/Stock (P.R.8/S), was an egg-adapted P.R.8 strain of Influenza A obtained from the National Institute for Medical Research, Mill Hill, in 1947. It has undergone 169 allantoic passages in this laboratory. For all absorption experiments the virus was purified by adsorption on to red cells and subsequent elution into saline. The strain P.R.8/X used in preliminary experiments was a mutant strain derived from P.R.8/S, and characterized by its poor Francis response after heating.

Serum.

Normal rabbit sera were used throughout. In the early experiments sera from two individual rabbits were used. For later work two pools of sera, Pool 1 from eleven rabbits and Pool 2 from nine rabbits, were used. Both these serum pools were inactivated at 56° C., Pool 1 for 45 minutes and Pool 2 for 15 minutes. Both were filtered through 0·74μ Gradocol membranes and stored at — 20° C.

Red blood cells.

Fowl cells from nine fowls were used usually in pools obtained from two to four fowls. They were stored at 4° C. as packed cells and washed before use. Cells older than seven days were not used. All titrations were carried out using fowl cells, but human Group O cells were used for most absorption experiments.

Filtration.

Gradocol membranes 0·74μ A.P.D. were used for preliminary filtration of sera and for clarification of virus-serum mixtures before their filtration through finer membranes.

Membranes of 0·2 to 0·17μ A.P.D. were used for removal of virus from mixtures. In all cases they were found to be effective in removing all traces of virus detectable by haemagglutination. They were, however, freely permeable to serum inhibitor. Filtration was by negative pressure up to 40 cm. Hg.

Cholera enzyme.

Receptor destroying enzyme (R.D.E.), was prepared according to the method of Burnet and Stone (1947), and freeze-dried. It was used in a menstruum of borate buffer pH 6·8 with 0·1 per cent calcium chloride. Virus titrations in the presence of R.D.E. were carried out in borate buffer pH 6·8 with 2·5 per cent sodium citrate.

Haemagglutination technique.

Titrations were performed by the method of Hirst and Pickels (1942), using a photoelectric densitometer. All red cell suspensions were standardized, so that invariably a 1 in 4 dilution gave the same galvanometer reading, which was accepted as the 50 per cent agglutination end-point; the approximate concentration was 1 per cent packed cells. For virus titrations the dilutions were made in 1 ml. volumes and 1 ml. of R.B.C. suspension added. For serum inhibition titrations serum dilutions were made in 0·5 ml. volumes, 4 A.Ds. of virus in 0·5 ml. were then added and finally 1 ml. of R.B.C. suspension. Tubes were mixed by inversion, and read 75 minutes from the time of mixing.

Interpolated titres were calculated from the readings above and below the 50 per cent end-point. Extrapolation from the graph of the titration was occasionally used when the curve justified the procedure. Titres are expressed as the reciprocal of the calculated final dilution of virus or serum at the 50 per cent agglutination end-point.

EXPERIMENTAL.

Preliminary Investigations.

Effect of heat inactivation of virus.

The relationship between duration of heating virus at 56° C., and its susceptibility to inhibition by normal rabbit serum was studied. Fig. 1 shows typical response curves for both the P.R.8/S and P.R.8/X strains. The change in the virus which gives rise to the Francis effect is progressive, the serum titre continuing to rise with increased time of heating as long as the virus retains sufficient haemagglutination activity to make possible the employment of a standard dose of 4 A.D. This effect was obtained regularly in all experiments. Some irregularity in the response is frequently observed during the first hour of heating, but this does not affect the general trend of the curves. Since the power of elution from red cells of both the virus strains is consistently and completely destroyed by heating at 56° C., for 30 minutes, progressive destruction of the virus enzyme would not appear to be an adequate explanation of the phenomenon.

Francis effect at 2° C.

Comparative titrations of rabbit serum inhibitor against living and heated viruses were carried out at room temperature and at 2° C. In each case the virus

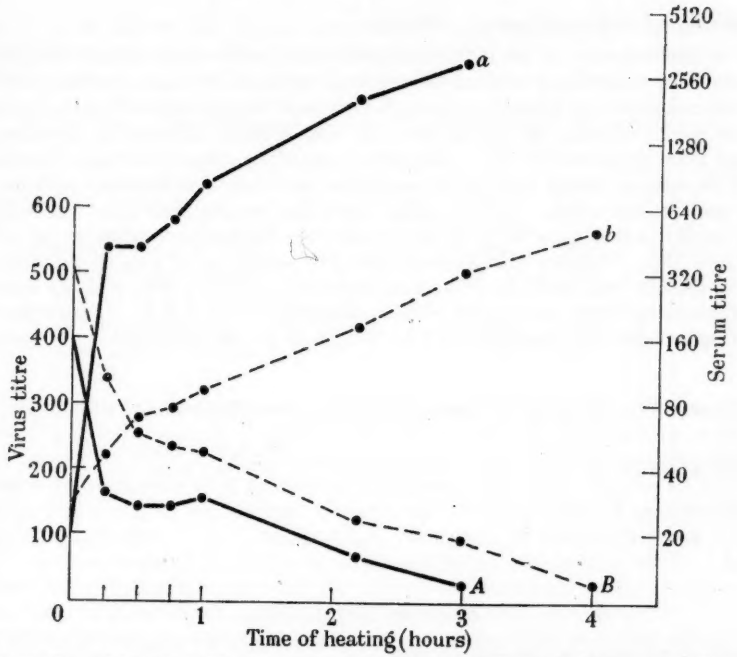

FIG. 1.—Effect of duration of heat treatment of virus on the Francis phenomenon. Curves A and B show virus titres; curves a and b show serum inhibitor titres; unbroken lines, virus strain P.R.8/S; broken lines, virus strain P.R.8/X.

dose (4 A.D.) was calculated from a preliminary virus titration at the respective temperature. The results summarized in Table I fully confirm previous reports that the Francis effect occurs equally well at either temperature. Exact correspondence between the H/L ratios would, of course, not be expected, as temperature affects both the speed of reactions between the reagents and the rate of cell sedimentation.

TABLE I.—*Comparison of Francis Effects at Room Temperature and at 2° C.*

Expt. No.	Serum.	Virus strain.	Serum inhibitor titres *v.* 4 A.D. virus. Room temperature. L.	H.	H/L.	2° C. L.	H.	H/L.
1	Rabbit 1	P.R.8/S	49	245	5·0	95	1280	13·5
		P.R.8/X	30	57	1·9	70	121	1·7
2	Rabbit 2	P.R.8/S	50	148	3·0	160	471	2·9
		P.R.8/X	20	77	3·8	40	238	6·0
3	Pool No. 2	P.R.8/S	40	214	5·3	140	349	2·5

L = living virus; H = heated virus.

Filterability of virus and serum inhibitor.

As a preliminary to absorption experiments with virus serum mixtures it was necessary to devise a method for the separation of free and absorbed inhibitor. This was achieved by filtration through Gradocol membranes of known porosity. Normal rabbit serum, 40 ml. of Pool 2, was filtered through a membrane of average pore diameter 0·74μ. Aliquots were then passed through membranes of A.P.D. 0·31μ, 0·24μ and 0·19μ and the four filtrates titrated against both living and heated virus. It is evident from the results shown in Table II that serum inhibitor will pass without detectable loss through membranes of A.P.Ds. down to 0·19μ. Tighter membranes were not tested, as it was found that virus was completely held back at 0·2μ, and that only about 2 per cent, as tested by haemagglutinin titres, passed at 0·24μ. Membranes of A.P.D. 0·2μ or less were accordingly used for recovery of free inhibitor in the absorption experiments.

TABLE II.—*Passage of Serum Inhibitor through Gradocol Membrane.*

Virus (4 A.D.).	Titres of serum filtrates.			
	0·74μ.	0·31μ.	0·24μ.	0·19μ.
P.R.8/S living	40	36	37	31
,, heated	238	234	261	266

Absorption Experiments.

Inhibitor absorption in absence of cells.

Burnet's hypothesis of a dynamic equilibrium demands that heated virus should possess greater affinity for inhibitor than living virus relative to their affinities for red cell receptors. In view of the extraordinary and progressive exaltation of the Francis effect on continued heating, it seemed possible that the heat treatment might increase the absolute affinity of virus for inhibitor quite irrespective of the presence of red cells in the reaction mixture. In such case the relative avidities of living and heated virus could readily be compared by titration of the filtrates of absorption mixtures for their residual inhibitory activity.

Three experiments designed to this end are summarized in Table III. In order to facilitate filtration the mixtures were passed in each case first through a coarse membrane, and then immediately thereafter through one of 0·17μ A.P.D. Filtrates were titrated against 4 A.D. heated virus. Experiment 1 shows that virus subjected to the usual heat treatment for demonstration of the Francis effect does in fact form a firm complex with inhibitor which is removable by filtration, whilst allowing free inhibitor to pass through. Experiment 2 shows that virus, even when completely inactivated so that it is no longer capable of causing any sign of red cell agglutination, can still absorb inhibitor. Experiment 3 compares the absorptive capacities of equal volumes of living and heated virus as a direct test of the thesis that heating may increase absorptive power. In this case the absorptions and filtrations were carried out at 2° C. to avoid enzymic destruction of inhibitor by the living virus. Clearly the living virus showed not less but greater absorptive power than the heated virus. Indeed, it must have absorbed nearly all the inhibitor present, for at the lowest dilution of filtrate

tested, 1 in 80, there was 71 per cent red cell agglutination compared with 79 per cent given by the 4 A.D. virus-saline control.

TABLE III.—*Absorption of Inhibitor by Living and Heated Viruses.*

Expt. No.	Absorbing virus P.R.8/S. Treatment.	Titre.	Dose. ml.	Dose. (A.D.)	Serum absorbed. Vol. ml.	Initial titre.	Residual titre.
1	Heated 56° C./30 min.	146	9	657	0·1	996	<400
2	Totally inactivated 56° C./ 7 hr.	0	5	0	0·5	387	178
3	Living untreated.	293	5	685	0·5	188	<80
	Heated 56° C./30 min.	89	5	160	0·5		97

The employment of equal volumes of living and heated virus for comparison of their absorptive powers is only justifiable on the assumption that with heat treatment all the virus elements present undergo a similar degree of degradation. An alternative possibility is that some virus may be totally destroyed and some partially degraded, in which case reduced absorptive power of a given volume would be inevitable. The Francis effect, however, is obtained when sera are titrated against standard 4 A.D. doses of both living and heated virus. Absorption tests were therefore made with the amounts of absorbing viruses equated both on the basis of their haemagglutinating activities and by volume.

Selection of dosage.—Because comparative absorption tests in which living virus is used must be done at a low temperature in order to obviate inhibitor destruction by enzyme action, the virus agglutination titres used for the calculation of absorption doses should ideally be those exhibited at the same low temperature. It was found, however, that the H/L virus titre ratios from titrations carried out at room temperature, and at 2° C. were almost identical. This was considered to justify the equating of absorbing doses on the basis of titrations at room temperature so as to reduce to a minimum the amount of work in the cold room.

TABLE IV.—*Ratios Heated/Living Virus Titres when Estimated at R.T. and at 2° C.*

Expt. No.	Virus strain.	H/L ratios. R.T.	2° C.
1	P.R.8/S	0·45	0·39
2	,,	0·64	0·61
3	,,	0·41	0·45
4	P.R.8/X	0·5	0·5
5	,,	0·39	0·46

An absorption experiment was carried out, using the following virus doses :

1. Living virus 1·7 ml. = 1600 A.D.
2. ,, ,, 0·43 ml. = 400 A.D.
3. Heated virus 1·7 ml. = 400 A.D.

Each of these was mixed with 0·5 ml. serum in a total reacting volume of 10 ml. and the respective filtrates obtained. The titration curves for residual inhibitor are shown in Fig. 2. At 400 A.D. dosage the curves of living and heated virus are practically superimposed, whereas the curves for equal volumes are widely separated. Thus A.D. for A.D. living and heated virus have exactly the same absorbing power for serum inhibitor, although the latter gives a pronounced Francis effect.

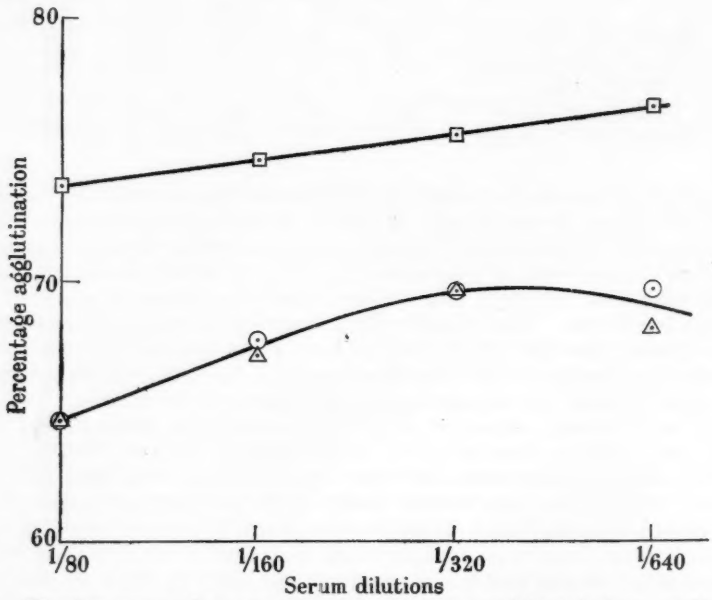

FIG. 2.—Titration curves of a serum absorbed with living and heated virus. ⊡ Living virus; absorption dose 1·7 ml. = 1600 A.D. ⊙ Living virus; absorption dose 0·43 ml. = 400 A.D. △ Heated virus; absorption dose 1·7 ml. = 400 A.D.

Inhibitor absorption in presence of red cells.

In spite of the equal absorbing potencies of living and heated virus in simple virus-serum mixtures, there remained the possibility that the two might differ in their relative avidities for red cell receptors, so that the dynamic equilibrium in serum inhibition titration mixtures would stabilize at different levels. The point was investigated by absorptions in the presence of agglutinable red blood cells. To weight the conditions in favour of the Burnet hypothesis the red cells were added to a concentration double that employed by us in all titration work. Absorption mixtures in the majority of experiments consisted of 0·5 ml. serum, 200 A.D. virus and 1 ml. packed R.B.Cs. in a total volume of 10 ml., the reagents being suitably diluted before mixing. All absorptions were done with chilled materials in the cold room at approximately 2° C. The reagents were mixed in the same order as in a normal Francis inhibitor titration, serum and virus being allowed 5 minutes' contact before the introduction of red cells. For the first

experiment pooled fowl cells were used; thereafter human group O cells were substituted as the large amounts required imposed too great a strain on the fowls available. After 15 minutes' further contact the cells were spun off and the supernatant fluids filtered through membranes of 0·2μ or 0·19μ A.P.D. in order to remove virus. At this point work was transferred from the cold room to the laboratory, where titrations of residual inhibitor were done against 4 A.D. of a suitable indicator virus, usually the same heated virus as was used for absorption. Fowl cells were used for all the titrations.

The results of four experiments, including a control experiment without admixture of red cells, are shown in Table V. The "units of inhibitor" are calculated on the basis that one unit neutralizes one A.D. of virus. At the 50 per cent titration end-point three out of four A.D. of the virus are neutralized, so that 2 ml. of the serum dilution at this end point contain 3 units of inhibitor. In all these experiments 0·5 ml. serum was absorbed, so that inhibitor content is given by the formula 0·5 × titre × 3/2. This unit is purely arbitrary, and the apparent potency of a serum will vary with the virus used for the titration; nevertheless it serves as a useful basis for the comparison of the absorption powers of viruses in any single experiment.

TABLE V.—*Absorption of Normal Rabbit Serum in Presence and Absence of R.B.C.*

		Absorbing virus.			Serum.			
Expt. No.	R.B.C.	Batch.	Nature.	Dose A.D.	Titre.	Units of inhibitor present.	Units of inhibitor absorbed.	Units absorbed per A.D. of virus.
1	Human	E/150	*Nil* (control)	—	640	480	—	—
			Living	200	88	66	414	2·1
			Heated	200	136	102	378	1·9
2	„	E/150	*Nil* (control)	—	560	420	—	—
			Living	200	160	120	300	3·0
			Heated	200	139	105	315	3·2
3	Fowl	E/166	*Nil* (control)	—	2308	1731	—	—
			Living	151	30	23	1708	11·3
			Heated	135	86	65	1666	12·3
4	Nil	E/158	*Nil* (control)	—	533	400	—	—
			Living	100	270	178	222	2·2
			Heated	100	270	178	222	2·2

For explanation of "Unit of Inhibitor" see text.

The close agreement between units of inhibitor absorbed by living and heated viruses shown in the last column of Table V is remarkable in view of the complexity of the experiments. The conclusion appears inescapable that, in the presence of red cells under conditions comparable with those obtaining in the mixtures of a Francis titration, equal haemagglutinating doses of living and heated virus exhibit not only equal avidity for serum inhibitor, but also equal relative avidities for inhibitor and the red cell receptors. As a final check Francis titrations were carried out in parallel with an absorption experiment, with the same serum and virus preparations, both at room temperature and at 2° C. Pronounced Francis effects were obtained in each case.

Further experiments were carried out in which aliquots of serum were absorbed with a given dose of living virus, with and without the presence of red cells.

The amounts of inhibitor absorbed were identical in one experiment and not significantly different in another. This indicates that the virus-inhibitor complex is not dissociated by the subsequent addition of red cells—a reaction which would be necessary in order to establish a dynamic equilibrium in Francis titrations. Of course, if the virus and cells were mixed first and the serum added subsequently, much less inhibitor would almost certainly be absorbed, for the order of adding reagents profoundly affects the results of inhibitor titrations. As, however, the addition of cells to previously mixed virus and serum dilutions is universally practised for serum titrations this point was not directly tested.

The time factor in inhibitor absorption.

The period of contact of virus and serum prior to the addition of red cells may, or may not, affect the magnitude of the Francis effect. This is because such contact always increases the serum titre against heated virus, sometimes to quite fantastic heights, whereas it may or may not increase the titre against living virus. This discrepancy in behaviour of different batches of living virus of the same strain has not been elucidated, but in any case cannot be the basis of the Francis effect; firstly because equivalent Francis effects may often be obtained with and without prior contact of virus and serum, secondly because reduction of combining speed from heat treatment would tend to give exactly the reverse effect, namely a lower inhibitor titre with heated virus than with living virus.

These time effects are illustrated by the experiments recorded in Tables VI and VII and Fig. 3. The two experiments in Table VI were comparative Francis titrations done at 2° C. Contact periods of 6 hours and 4 hours raised the H/L serum titre ratios to a remarkable degree, for in each case the L titres remained unaffected. Fig. 3 graphically records the result of an experiment of like nature also at 2° C. in which the effect of time of contact was followed over a period of $3\frac{1}{2}$ hours by setting up the virus-serum dilutions in quadruplicate and adding red cells to the sets at intervals. Here the living and heated virus curves run roughly parallel, giving approximately equal H/L serum titre ratios throughout. Table VII gives the result of an absorption test carried out at 2° C. in parallel with the Francis titration experiments and with the same materials. The absorption mixtures consisted of 0·5 ml. serum and 100 A.D. virus in 10 ml. total volume, red cells not being present. Aliquots were filtered immediately and after two hours' contact, period of contact being taken as the time elapsing between mixing reagents and completion of filtration. Filtrates were titrated against two different batches of heated indicator virus. The differences between the titres of serum absorbed by living and heated virus at corresponding times are not significant.

TABLE VI.—*Effect of Time of Contact of Virus and Serum on the Francis Effect.*

Expt. No.	R.B.C. added to serum-virus mixtures.	Serum titres v 4 A.D. virus. L.	Serum titres v 4 A.D. virus. H.	Ratio H/L.
1	Immediately	95	960	10·1
	After 6 hours	80	>2560	>32·0
2	Immediately	61	640	10·5
	After 4 hours	65	>10,240	>158·0

TABLE VII.—*Influence of Time of Contact on Absorption of Inhibitor by Living and Heated Virus.*

Absorbing virus.			Titres of absorbed serum against indicator viruses.	
	Dose A.D.	Time of contact (minutes).	H1. 4 A.D.	H2. 4 A.D.
Nil (control)	—	—	533	1002
Living	100	3	270	463
,,	100	120	143	385
Heated	100	5	270	501
,,	100	120	181	349

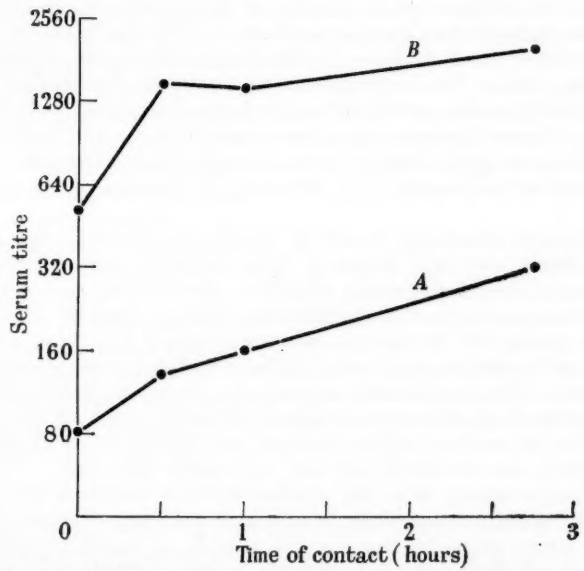

FIG. 3.—Effect of time of contact of virus and serum on the Francis effect at 2° C. A, Serum titres against 4 A.D. living virus; B, serum titres against 4 A.D. heated virus.

DISCUSSION.

In the experiments reported living and heated virus, A.D. for A.D. behaved identically, both in respect of combining capacity for serum inhibitor and also rate of combination. It must be emphasized, however, that all absorptions were done within the range of virus-serum proportions required to relate the occurrences to the mechanism of the Francis phenomenon. In order to elucidate completely the dynamics of virus-inhibitor reactions it would be necessary to carry out multiple absorptions on the chess-board pattern, testing each of a large range of virus quantities against each of a similarly extended range of serum quantities, and furthermore making in each test replicate titrations of the free

inhibitor at various time intervals. Our resources hitherto have not been adequate for a task of such magnitude. However, from the present limited investigation it is clear that neither of the two hypotheses which have been previously advanced to account for the Francis effect is valid.

It is impossible that enzymic destruction of inhibitor by living virus, if it occurs at all at 2° C., should be as great as or greater than that occurring at room temperature. Yet the Francis effect is often more pronounced at the lower temperature. Also, not all virus strains possessing demonstrable enzyme activity show the increased sensitivity to inhibitor on heating. Again, the inhibitor sensitivity continues to increase with prolonged heating long after all demonstrable enzymic activity has been lost.

Burnet's hypothesis of a dynamic equilibrium rests upon the assumption that the degree of haemagglutination inhibition in a serum titration mixture is a measure of the relative absorptive affinities of the virus for inhibitor and red cell receptors. He suggests that on heating a virus " changes in its surface configuration are produced which increase its absorptive affinity for various mucoids " (Anderson *et al.*, 1948). We believe that heat treatment does in fact alter surface configuration, but that this is without appreciable effect upon its relative absorptive affinities. Hence the equivalent behaviour of living and heated virus with the same number of agglutinating doses of each, contrasted with the reduced combining power of the heated virus when equal amounts of the virus material are used.

The retention of absorbing power by totally inactivated virus suggests the possibility of there being two types of virus receptors with different grades of heat lability and different combining affinities. Such a dual receptor hypothesis, however, also runs counter to our experimental findings, because whatever affinity differences are postulated for the two receptor types, it follows logically that the living virus should combine with more inhibitor and at greater speed than should the heated virus. The phenomenon is probably based upon the well-established fact that in all serological reactions certain threshold doses of the reagents are required in order to produce visible changes, and that in different reactions with the same reagents the thresholds are not necessarily the same. " Total inactivation " may imply merely that the number of virus receptors has fallen below the required threshold, or that their distribution has been changed, so that they can no longer clump red cells, but are still capable of combining with inhibitor.

A hypothesis which we consider adequate to explain all the facts so far elicited is one which might be termed " the hypothesis of steric hindrance." We suggest that heating alters the surface configuration of the virus so that a given amount of inhibitor will blanket the combining groups to an extent sufficient for haemagglutination neutralization, whilst the same amount of inhibitor is insufficient to blanket living virus to the same extent. It is inconceivable that living virus, treated with such a critical dose of serum and still capable of causing haemagglutination should be unable to absorb more inhibitor; obviously it can do so because it is possible with sufficient serum to neutralize its haemagglutination activity completely. What our experiments have shown is that heated virus continues to absorb inhibitor at approximately the same rate long after the neutralization end-point has been reached. This presents no serious intellectual difficulty for, even assuming complete chemical identity of red cell receptor substrate and inhibitor, the former exists as part of the complex surface structure

of the relatively enormous red cell, whilst the latter is a serum constituent, probably of molecular size. The siting of the red cell receptors over the cell surface requires a complementary spatial arrangement of recepors on the surface of the virus for haemagglutination to occur. On distortion of this spatial arrangement any combined inhibitor may well prevent the apposition of adjacent free receptors to reactive cell sites, as well as neutralizing the virus receptors to which it is directly united. The distortion, however, may be insufficient to interfere with the approach and union of further molecules of inhibitor.

This hypothesis conforms to what is known to occur during the earlier stages of heat denaturation of many proteins. According to Astbury (1939) there is an unfolding of the polypeptide chains and the work of Pauling and Campbell (1942) on the artificial production of antibodies was based on this particular effect. Influenza virus consists largely of nucleoprotein, and it seems likely that the virus receptors form a part of protein complexes at the surfaces of the elementary bodies, especially in view of the closely associated enzymic activity. Unfolding of protein molecules at the surface would inevitably cause structural distortion, but would not necessarily destroy combining power for certain substrates.

We do not claim that the maximum absorptive powers of living and heated virus at the saturation point are equal; in fact we have reason to believe from the results of further experiments that this is not so. Any such differences at or near the saturation point, however, can have nothing to do with the mechanism of the Francis phenomenon.

It should be explicitly stated that the work reported in this paper has been confined to the interaction of a single strain of Influenza A virus and normal rabbit serum. Other strains may behave differently. Also species differences in the content and behaviour of serum inhibitory substances have been clearly demonstrated (Burnet and McCrea, 1946; Smith and Westwood, 1949). Nevertheless, it seems improbable that a multiplicity of mechanisms exist for a phenomenon so widely distributed amongst the influenza viruses as is the Francis phenomenon, and we suggest that the explanation offered is probably applicable both to other strains and other sera.

SUMMARY.

Prolonged heating of Influenza A virus causes a progressive degradation, which results in a progressive increase of the Francis effect as long as any haemagglutination activity remains.

The Francis effect is demonstrable equally at room temperature and at 2° C. The H/L titre ratios of a virus are the same at the two temperatures.

Heat treatment of virus, sufficient to convert it to a good indicator of the Francis effect, does not appreciably affect the power of a given number of agglutinating doses to absorb serum inhibitor. Over a wide range of dosage both total combining capacity and rate of combination remain unchanged. The situation is not affected by the presence of agglutinable red blood cells in the reaction mixtures.

The significance of these findings is discussed, and a new hypothesis is presented to explain the mechanism of the Francis phenomenon.

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PENICILLINASE ADAPTATION IN *B. CEREUS*: ADAPTIVE ENZYME FORMATION IN THE ABSENCE OF FREE SUBSTRATE.

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THE apparently enzymic nature of various penicillin " inactivators " produced by a wide range of penicillin-resistant micro-organisms has been observed by a number of workers (see excellent review by Chain, Florey, Heatley and Jennings, 1949). The term " penicillinase " has, however, been used rather loosely to cover most cases where penicillin has been biologically inactivated by means of some sort of enzymic process, and few biochemical studies of the phenomenon have been undertaken. Foster (1945) observed that a cell-free preparation from *B. subtilis* inactivated penicillin with the formation of acid. The formation of an acidic group with pK 4·7 by the action of penicillinase from a strain of *B. cereus* on penicillin was confirmed by Benedict, Schmidt and Coghill (1945) ; and, later, Henry and Housewright (1947) showed conclusively, by measuring CO_2 evolution from a CO_2-bicarbonate buffer manometrically, that the penicillinase in a broth culture filtrate of *B. cereus* destroyed penicillin by quantitative hydrolysis to penicilloic acid, with the formation of one acid equivalent. It still, of course, remains to be shown that enzymic penicillin inactivators from other sources destroy penicillin in a similar manner.

The fact that penicillinase from some strains of bacteria behaves as an adaptive enzyme was first observed by Duthie (1944), who, using a strain of *B. subtilis*, found that addition of penicillin to the culture media—even after cessation of growth—produced up to 30-fold increase in yield of the enzyme. A similar adaptive effect has been reported by Lepage, Morgan and Campbell (1946) for strains of *B. megatherium* and *B. cereus*, and later again by Duthie (1947) for strains of *B. licheniformis*. Housewright and Henry (1947) confirmed the marked effect of penicillin on penicillinase production by *B. cereus*.

METHODS.

Organism.—*B. cereus* NRRL B-569 from Dr. R. D. Housewright.

Cell suspensions.—Prepared from 20-hr. growth at 35° in tryptic meat broth aerated by shaking, inoculated with one drop of a spore suspension prepared by heating 50 ml. of a 48-hr. culture in casein hydrolysate yeast extract medium (Gladstone and Fildes, 1940) for 1 hr. at 60°, washing the cells and resuspending

in 20 ml. water. Cells were spun down, washed once in water and made up to standard strength (10 mg./ml. or 20 mg./ml.) in water. Cell concentrations were measured by opacity, using a Spekker absorptiometer and a standard opacity/dry weight curve prepared as described for *Bact. coli* (Pollock and Wainwright, 1948).

Penicillinase activities were measured manometrically at 30° in a CO_2-bicarbonate buffer at pH 7·0, as described by Housewright and Henry (1947). One ml. or 2 ml. samples of the enzyme preparation were added to 0·5 ml. of 0·043 M sodium bicarbonate in the main compartment, and made up to 2·5 ml. with water. 0·1 ml. of penicillin solution 100,000 units/ml., 0·1 ml. of 0·043 M sodium bicarbonate and 0·3 ml. water were put in the side bulb. The manometers were gassed with 5 per cent CO_2 in N_2. After equilibration for 20 min. the contents of the side-bulb were tipped into the main flask. After 2 to 5 minutes, a constant rate of gas evolution was established. Penicillinase activities were assayed by measuring the rate of CO_2 production over the linear phase which persisted until about 75 per cent of the penicillin was destroyed. Even with enzyme preparations of very low activity, measurements were not continued for longer than 30 min.

All values were corrected for CO_2 retention caused by substances such as phosphate, amino-acids, etc., present in the sample. The CO_2 retention was calculated by adding a known amount of pure tartaric acid (dried in the side-bulbs) to samples of the various media, in presence of the CO_2-bicarbonate buffer, and measuring the per cent reduction in gas evolution compared to that from bicarbonate alone. It was confirmed that such substances causing CO_2 retention in no way affected the linearity of the reaction.

Penicillinase production was followed in M/50 glucose, M/600 $MgSO_4$, M/50 phosphate buffer pH 7·0, in the presence of varying quantities of penicillin up to 10,000 units/ml. and with or without 0·8 per cent "Vitamin-free" casein hydrolysate (Ashe), supplemented with M/12,500 tryptophan and M/5000 cystine. The cell concentration varied from 0·5 to 2·0 g./ml., and the suspensions were incubated in conical flasks (usually 20 ml. in 100 ml. flasks) at 35° on a shaker (100/min., amplitude 10 cm.). Samples of 3·0 ml. were removed for assay, and added to 0·6 ml. of M/200 oxine in order to stop further enzyme production. Except when specifically mentioned otherwise cells were not separated from medium, so that enzyme activities represent the combined extra- and endo-cellular fractions present in the whole sample. These were usually assayed within 3 to 4 hr., but had, occasionally, to be left overnight (18 hr.) at + 2° before testing. It was confirmed that there is no loss of activity of such preparations, in the presence of M/1200 oxine, for at least 3 hr. at 35° and 18 hr. at + 2°. Results are expressed as μl. CO_2 released per ml. per hr. ("Total penicillinase") or μl. CO_2 per hr. per mg. dry weight of cells ("Q penicillinase").

Penicillin used was the pure sodium salt of penicillin G (benzylpenicillin) prepared by Glaxo Laboratories Ltd. Dilutions were made in M/100 phosphate buffer, pH 7·0.

Penicilloic acid was prepared by alkaline hydrolysis of sodium benzylpenicillin, using the method described in Clarke, Johnson and Robinson (1949). Attempts to obtain a crystalline product, however, failed, and a 30 per cent aqueous solution still showed antibiotic activity on *B. subtilis* layer plates several days after completion of hydrolysis. It was therefore reprecipitated with acid, freeze-dried

and twice dissolved in methanol on a water bath and evaporated down to dryness. The resulting product showed no antibiotic activity, and therefore contained less than 1 part per 10^6 of penicillin. Although it is possible that this amorphous product contained an appreciable amount of benzyl-penilloic acid, it can be regarded as consisting mainly (if not entirely) of benzylpenicilloic acid, and as being for all intents and purposes free from penicillin.

Growth was always measured by opacity estimation in a Spekker absorptiometer, and expressed as mg. dry bacterial wt./ml. after reference to the standard opacity/dry weight calibration curve.

RESULTS.

Preliminary.

The findings of Henry and Housewright (1947) regarding the pH/activity curve of penicillinase, the proportionality between rate of CO_2 evolution and enzyme concentration over a wide range, and the stimulatory effect of penicillin on penicillinase formation during growth in broth, were all confirmed. A small "basal" penicillinase activity was always detectable even if the cells were grown in the absence of added penicillin ; while in the presence of 500 units/ml. enzyme yield was increased over 40-fold.

It was then shown that penicillinase adaptation would occur rapidly without growth in the presence of 10,000 units/ml. penicillin with M/50 glucose, M/10 phosphate buffer and M/600 $MgSO_4$ only, using washed cells at a concentration of 0·6 mg./ml. Apart from the penicillin itself no N source was added, and opacity measurements showed no change over the $2\frac{1}{2}$ hr. of the experiment. The adaptation curve obtained (Fig. 1) is of the upward-sweeping, " logarithmic " type which occurs in other microbial enzyme adaptations.

Since penicillinase activities were usually measured using samples containing both intact cells and glucose, it would clearly have led to inaccurate results if there had been any marked gaseous change (e.g. due to anaërobic glycolysis) in the manometers before adding the pencillin, particularly if the penicillin had influenced the rate of any such change. In practice, however, it was found that gas changes before tipping were insignificantly small, and were therefore ignored from the point of view of estimating penicillinase activities. Moreover, penicillin at the concentration used for enzyme assay (3300 units/ml.) had no significant effect on O_2 uptake or on CO_2 output aërobically or anaërobically by cell suspensions using glucose as substrate.

Penicillin-sensitivity growth tests in tryptic meat broth showed the organism to be " intrinsically " fairly sensitive. An inoculum of 1 drop of a 10^{-7} dilution of an 18-hr. broth culture was completely inhibited by 1·5 units/ml. penicillin, but grew in 0·3 units/ml. Heavy inocula (e.g. up to 50 µg./ml.) would grow in 10,000 units/ml. within 24 hr. It is clear, therefore, that the organism's resistance to penicillin is of the " penicillinase " type (Spink and Ferris, 1947 ; Bondi and Dietz, 1948), and probably exists only in so far as it is able to destroy penicillin with the enzyme produced.

Extracellular production of penicillinase.

The penicillinase of B-569 has been described (Benedict *et al.*, 1945) as extracellular. By comparing activities of whole cultures or suspensions with those

of the supernatant fluid after centrifuging off the cells, it was indeed found that, with 48-hr. cultures, about 70 per cent of the enzyme activity was extracellular. During the early phases of enzyme production, however, the extracellular fraction was often much lower. For instance, during a $2\frac{1}{2}$-hr. period of adaptation of washed cells in penicillin (as illustrated in Fig. 1) the extracellular fraction varied from 0 to only 38·5 per cent, and in some experiments never exceeded 6 per cent. The process appears to be some active form of enzyme secretion, since there was

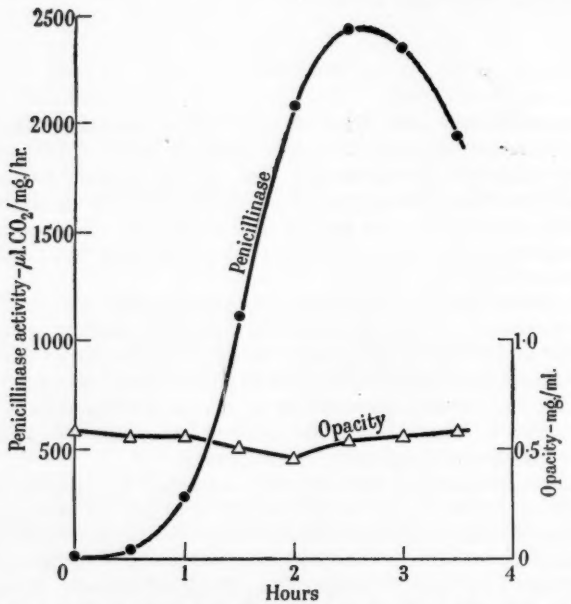

FIG. 1.—Penicillinase production by cells of *B. cereus* without added N source and in the absence of growth. Medium : M/50 glucose, M/50 phosphate buffer pH 7·0, M/600 $MgSO_4$. 35°, shaken in air.

no evidence at all of any cell lysis during this period, and addition of M/1200 oxine inhibited both further enzyme production and increase in the extracellular fraction.

Effect of oxine (*8-hydroxyquinoline*).

It has been found that suitable concentrations of oxine would inhibit nitratase adaptation in *Bact. coli*, without affecting the action of nitratase itself (Wainwright and Pollock, 1949). The same is true for pencillinase adaptation of *B. cereus* B 569 (Fig. 2) : M/1200 oxine completely inhibited further adaptation at 35°, while even double that concentration was found to have no significant effect whatever on the enzyme activity itself. Addition of M/1200 oxine to samples was therefore an ideal method for obtaining estimations of penicillinase activities at definite times during the period of enzyme production.

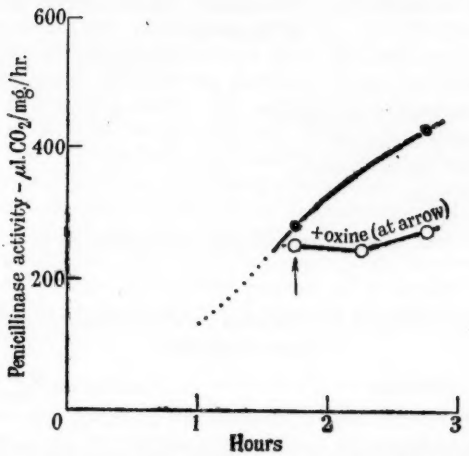

FIG. 2.—Inhibition of penicillinase production of *B. cereus* by M/1200 oxine. Medium : M/50 glucose, M/50 phosphate buffer pH 7·0, M/600 $MgSO_4$ + penicillin 10,000 units/ml. 1 mg./ml. cells. 35°, shaken in air. Open circles : M/1200 oxine added at 1¼ hr. and samples withdrawn at once, and after further ½ hr. and 1 hr. Filled circles : No oxine added. Samples withdrawn at 1¼ hr. and 2¼ hr. Cooled to 0° as rapidly as possible, and kept at 0° until assayed.

Effect of non-specific factors on penicillinase adaptation.

Oxygen.—Table I shows the effect of degrees of aëration on penicillinase adaptation with 10,000 units penicillin/ml. in the absence of added amino-acids. It is obvious that full aëration is necessary for maximal enzyme production, and it was repeatedly found that no significant amount was formed in the absence of O_2.

Mg.—Frequently no effect was found on the addition of M/600 $MgSO_4$, but since on some occasions stimulation of enzyme production occurred, Mg^{++} was added in all experiments.

Glucose was found to cause up to a 4-fold increase in the amount of enzyme produced in the absence of added N source and in the presence of 10,000 unit/ml. penicillin. The maximum effect occurred at a concentration of M/500. The action of glucose on penicillinase production is not, however, a simple one, and will be dealt with in more detail in a subsequent publication.

Temperature, pH.—No significant amount of enzyme was formed in 2 hr. at 18°, and about 4 times as much enzyme was produced at 35° as at 30°. Enzyme adaptation was maximal at pH 7·0, while below 6·5 it fell off rapidly.

Amino-acids, in the form of purified casein hydrolysate (Ashe) with added tryptophan and cystine, caused considerable stimulation of both basal penicillinase production (i.e. in the absence of penicillin) and penicillinase adaptation (with 10,000 units/ml. penicillin)—see Table II. No growth occurred in the presence of penicillin, even with added amino-acids, over the period of the experiment. Extent of stimulation by amino-acids was variable, and in some experiments amounted to a 6-fold increase in enzyme production.

TABLE I.—*Effect of Aëration on Penicillinase Adaptation of Washed Suspensions of B. cereus.*

Conditions.	Q penicillinase after 110 min. incubation at 35°.
In 6 in. × ¾ in. tube filled with argon	5
In 6 in. × ¾ in. tube open to air	9
In 50 ml. conical flask open to air	38
Ditto shaken at 100/min.	108
Ditto shaken at 350/min.	96

All vessels contained 5 ml. fluid with M/50 glucose, M/50 phosphate buffer pH 7·0, M/600 $MgSO_4$ and cells at 1 mg./ml.

TABLE II.—*Effect of Casein Hydrolysate on Pencillinase Production with and without Penicillin.*

Additions.	Q penicillinase after incubation for 100 min. at 35°.
Nil	6
10,000 units/ml. penicillin	1510
0·8 per cent casein hydrolysate	47
10,000 units/ml. penicillin + 0·8 per cent casein hydrolysate	2450

All flasks contained M50 glucose, M/50 phosphate buffer pH 7·0, M/600 $MgSO_4$ and initial cell concentration of 1 mg./ml. Casein hydrolysate was supplemented with tryptophan and cystine (see Methods).

TABLE III.—*Effect of Penicilloic Acid on Penicillinase Adaptation.*

Additions.	Q penicillinase after incubation for 2½ hr. at 35°.
Nil	10
2×10^{-7} M penicillin	790
2×10^{-2} M penicilloic acid	0
2×10^{-7} M penicilloic acid	10
2×10^{-7} M penicillin + 2×10^{-2} M penicilloic acid	905
2×10^{-7} M penicillin + 2×10^{-7} M penicilloic acid	640

All flasks contained 10 ml. of M/50 glucose, M/50 phosphate buffer, M/600 $MgSO_4$ and 1 mg./ml. washed cells of *B. cereus*.

TABLE IV.—*Effect of Penicillin Concentration on Penicillinase Adaptation.*

Penicillin conc. (units/ml.).	Q penicillinase after incubation for 2½ hr. at 35°.
0	46
0·0008	80
0·004	307
0·02	815
0·1	1190
10,000	133

All flasks contained M/50 glucose, M/50 phosphate buffer pH 7·0, M/600 $MgSO_4$ and cells (1 mg./ml.).

Specificity of stimulation of enzyme production by penicillin.

M/20 benzylalanylalanine (kindly supplied by Mr. H. Proom, of Burroughs Wellcome Laboratories, Beckenham) and M/25 glutathione, substances thought to be somewhat similar in structure to penicillin, were tested for ability to stimulate penicillinase production under the standard conditions (no added amino-acids) over a period of 2 hr. at 35°, but no stimulation occurred in either case. The first preparation of penicilloic acid (M/50) similarly tested was found to be nearly as active as penicillin in promoting adaptation. However, this had been prepared simply by alkaline hydrolysis of benzylpenicillin—without reprecipitation or methanol treatment—and biological assay showed it to contain about 1 : 100,000 active penicillin. This impurity was shown to be entirely responsible for the result (see below). A purified sample of penicilloic acid prepared as described in " Methods " was shown to be without significant activity on enzyme production (see Table III).

Effect of penicillin concentration.

Table IV shows the effect of different penicillin concentrations on penicillinase formation after 2½ hr. incubation in the standard glucose-Mg medium. It can be seen that a concentration of only ·004 units/ml. ($\triangleq 8 \times 10^{-9}$ M) caused a 7-fold increase in enzyme production over the control without penicillin, and even a concentration of ·0008 units/ml. produced significant stimulation. Moreover, the concentration previously employed (10^4 units/ml.) was actually inhibitory.

This result, though it adequately explains the effect first found with impure preparations of penicilloic acid, was unexpected. It was further found that samples of cells taken during adaptation in pencillin-glucose media, washed and resuspended in the same medium without penicillin, continued to form penicillinase. This was at first thought to be due to inadequate washing, but two further washings (five in all) only slightly reduced the rate of enzyme formation.

Enzyme formation by penicillin-treated cells after removal of penicillin.

It was eventually discovered that simple treatment of washed cells with penicillin for a short period at 0° conferred on them, even after thorough washing, the ability to form large amounts of penicillinase when subsequently incubated in the absence of penicillin (Fig. 3). This enzyme formation following penicillin-treatment was not qualitatively different from that occurring in the presence of penicillin. That is : it was stimulated by added amino-acids, it did not occur at all anaërobically, and it was inhibited by M/1200 oxine. The shape of the production curve, however, was different. Instead of being of the " logarithmic " type usually associated with enzyme adaptation, it had a short, but variable, lag phase (usually about 1 hr.) followed by a linear phase and, finally, a stationary phase which was, in some experiments, followed by a phase of decline —i.e. actual loss in enzyme activity.

Effect of added N source.—Although addition of casein-hydrolysate allowed cell growth to occur (up to a 3-fold increase in opacity during all except long-term experiments), this only had the effect of increasing the rate of enzyme formation and did not affect the linearity of the middle portion of the curve. In fact, the " tailing away " effect after the linear phase was shown conclusively to be due

to shortage of glucose and casein hydrolysate. Fig. 4 shows that the linear phase could be restored by replenishment of glucose and a source of amino-acids at the point where enzyme production was failing. In experiments where the medium was replenished earlier, no initial decrease in enzyme production occurred and

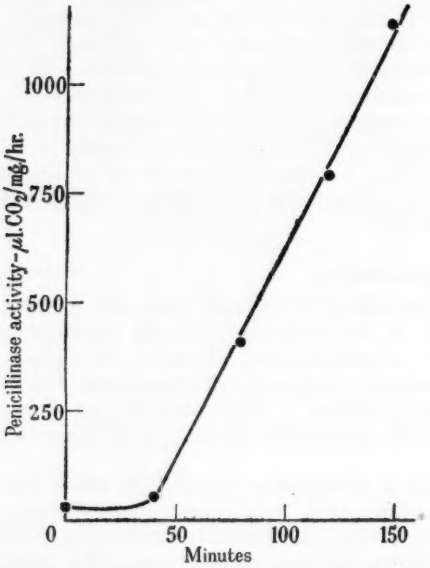

FIG. 3.—Linear production of penicillinase in M/50 glucose, M/50 phosphate buffer pH 7·0 and M/600 $MgSO_4$ by *B. cereus* cells following treatment with 1 unit/ml. penicillin. 35°, shaken in air. 1 mg./ml. cells.

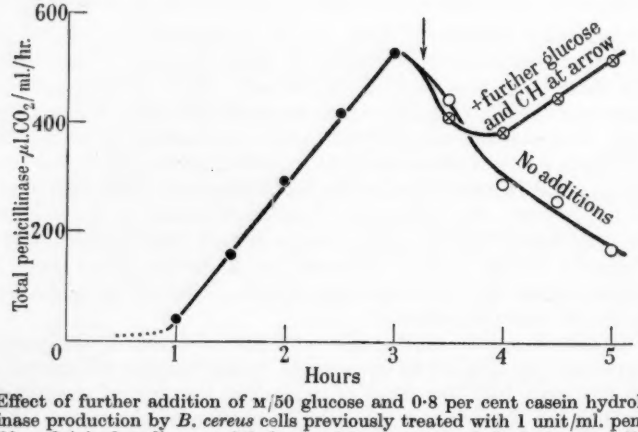

FIG. 4.—Effect of further addition of M/50 glucose and 0·8 per cent casein hydrolysate on penicillinase production by *B. cereus* cells previously treated with 1 unit/ml. penicillin for 1 hr. at 0°. Original medium : M/50 glucose, 0·8 per cent casein hydrolysate, M/600 $MgSO_4$ and M/50 phosphate buffer pH 7·0. 35°, shaken in air. Initial cell conc. 1 mg./ml.

the linear phase continued without interruption. This constant rate of penicillinase production could, in fact, be prolonged apparently indefinitely by suitable conditions (see below). The decrease in enzyme activity after 3 hr. shown in the culture without replenishment of glucose and casein hydrolysate has not been further studied. It is clearly not due to simple enzyme instability, since it has

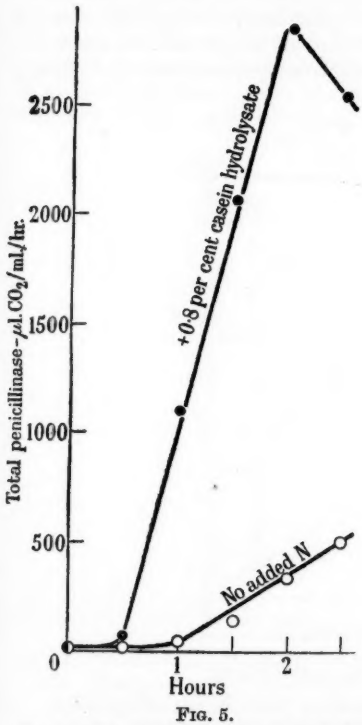

FIG. 5.

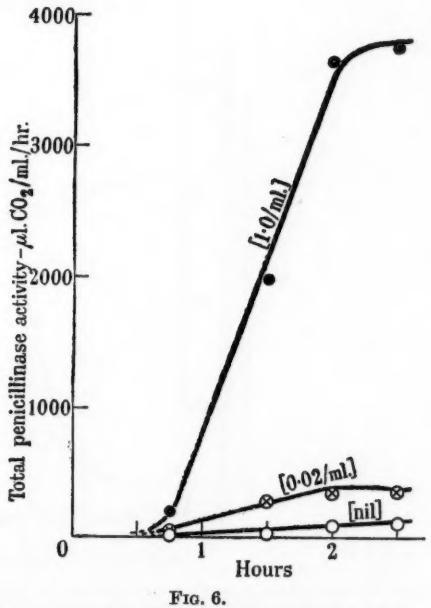

FIG. 6.

FIG. 5.—Effect of 0·8 per cent casein hydrolysate on rate of penicillinase production in M/50 glucose + M/600 $MgSO_4$ + M/50 phosphate buffer pH 7·0 by *B. cereus* cells previously treated with 1 unit/ml. penicillin. 35°, shaken in air. Initial cell conc. 1 mg./ml.

FIG. 6.—Rates of penicillinase production by *B. cereus* in glucose + casein hydrolysate after treatment of cells for 1 hr. at 0° with different concentrations of penicillin. Figures in brackets on the curves indicate concentrations of penicillin (units/ml.) used for pretreatment. 35°, shaken in air. Initial cell conc. 1 mg./ml.

been repeatedly shown that no loss of activity occurs at 35° in the presence of M/1200 oxine. It must therefore be connected with cell metabolism, and may be related to the phenomenon of enzyme "interaction" demonstrated by Spiegelman and Reiner (1947), which is said to occur more readily under conditions of N starvation.

Fig. 5 shows typical curves of enzyme formation, with and without added casein hydrolysate, by cells previously treated with 1 unit/ml. penicillin in M/100 phosphate buffer at 0° for 1 hr. followed by three thorough washings. This

stimulatory effect of amino-acids meant more accurate comparative results in the study of the pretreatment effect, and all subsequent experiments were done with added 0·8 per cent casein hydrolysate.

Effect of penicillin concentration.—The rate of penicillinase production after penicillin treatment was found to increase with increase in the concentration of penicillin used—up to a maximum of 1 unit/ml. A typical series of curves showing this effect is illustrated in Fig. 6. It was not practicable to test the effect of all the different concentrations of penicillin on the same suspension on the same day—and variability in adaptability between different suspensions and falling

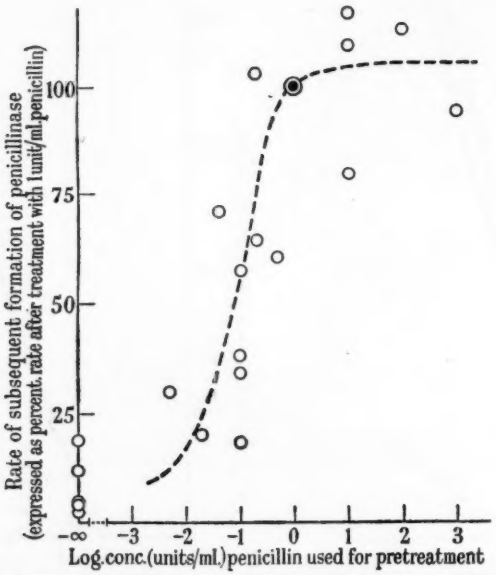

FIG. 7.—Relation between concentration of penicillin used for pretreatment of cells of *B. cereus* (for 1 hr. at 0°) and subsequent rate of penicillinase production in glucose + casein hydrolysate. 35°, shaken in air. Initial cell conc. 1 mg./ml.

off of adaptability of suspensions on storage precluded the comparison of absolute rates of enzyme formation between different experiments. If, however, rates of enzyme formation in all experiments are expressed as percentages of those obtained (always measured over the linear phase), using penicillin at 1 unit/ml., and plotted against penicillin concentration used, Fig. 7 is obtained. It can be seen that maximal rate of enzyme production occurs when the penicillin used for pretreatment was at a concentration of about 1 unit/ml., and half-maximal production at about 0·1 unit/ml. Maximal production rate is from 6 to 50 times that of the control without penicillin treatment.

Other factors.—It was found that this penicillin effect occurred very rapidly. Fig. 8 shows the comparative rates of penicillinase production measured over the linear phase after treatment with 1 unit/ml. penicillin at 0° for different lengths

of time. Washed cells, precooled to 0°, were added to M/100 phosphate buffer pH 7·0 containing 1 unit/ml. penicillin in an iced water-bath, to final cell concentration of 1 mg./ml. Samples were removed after 10, 60 and 180 minutes and the cells centrifuged down as rapidly as possible (10 min.), washed four times, resuspended to standard opacity (10 mg./ml.), and their rates of penicillinase formation at 35° in the glucose/casein hydrolysate medium compared to that of a similarly washed sample of cells treated for 10 min. in M/100 phosphate without penicillin. The rate of enzyme formation after only 10 min. treatment (to which perhaps should be added the time taken in the first centrifugation) was 66 per cent of that after 1 hr. treatment, and incubation for 3 hr. increased the rate only 35 per cent above that attained after 1 hr.

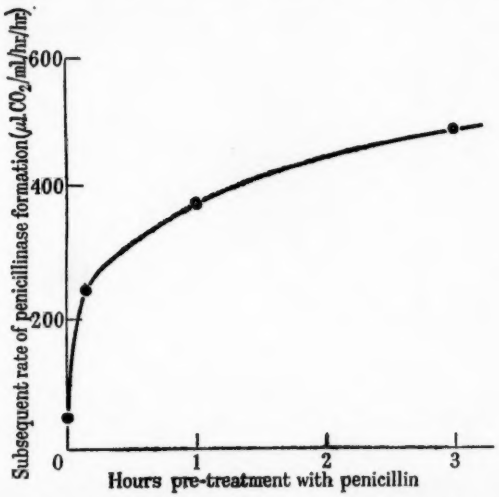

FIG. 8.—Relation between length of treatment of *B. cereus* cells at 0° with 1 unit/ml. penicillin and subsequent rate of penicillinase production in glucose + casein hydrolysate. 35°, shaken in air. Initial cell conc. 1 mg./ml.

The effect of penicillin treatment was more marked at 0° than at 35°. The rate of enzyme formation after treatment with penicillin for 1 hr. at 0° was $2\frac{1}{2}$ times more rapid than after treatment at 35°. The effect of penicillin treatment was, however, not altered by the concentration of cells used during treatment—at least within the range of 1 to 5 mg./ml.

Effect of continued growth without penicillin.—By continually making 1 : 2 dilutions of a culture in fresh glucose-casein hydrolysate medium it was possible to follow penicillinase production and growth, using the heavy inoculum of 0·5 mg./ml. almost indefinitely. Fig. 9 illustrates an experiment where the cells were pretreated for 1 hr. at 0° with M/100 phosphate buffer containing 0 and 1 units/ml. penicillin respectively (followed by three washings), and were maintained in the logarithmic phase for about 3 hr. by half-hourly 1 : 2 dilutions in fresh medium. Growth opacity and penicillinase activities were measured every $\frac{1}{2}$-hr. and the figures multiplied by the total dilution factor to give the results

in terms of 1 ml. of original medium. It is clear that: (*a*) Growth rate of the two lots of cells (untreated and treated with penicillin) is identical; (*b*) after the lag phase of 1 hr., penicillinase production by treated cells is approximately linear for the whole period of 5½ hr. of the experiment, and strictly linear up to 3½ hr. The relatively slight deviations from the straight line in the last 2 hr. are probably due to a combination of the high dilution correction (64-fold at 5½ hr.), and the increasing fraction of basal penicillinase that is being formed all the time in an amount roughly proportional to the total amount of growth; and (*c*) the Q penicillinase of the treated cells rises rapidly to a maximum at 2½ hr.

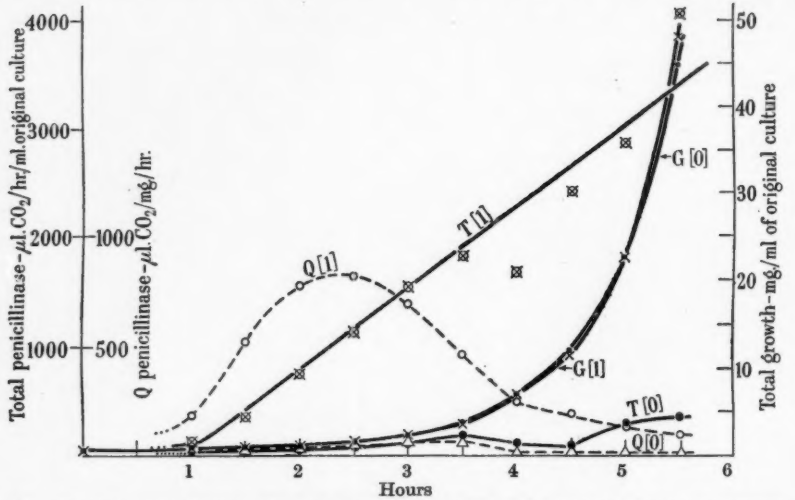

FIG. 9.—Continuous production of penicillinase during growth of *B. cereus* following treatment of cells with penicillin at 1 unit/ml., compared to control without penicillin pretreatment.

Q = Q penicillinase (µl. CO_2/mg. cells/hr.)
T = Total penicillinase (µl. CO_2/hr.)
G = Growth of cells (opacity measurements)
} Expressed as per ml. original medium.
[1] = Penicillin-treated cells (1 unit/ml.).
[0] = Untreated cells.

Medium: M/50 glucose, M/600 $MgSO_4$ + 0·8 per cent casein hydrolysate. 35°, shaken in air. Both cultures were diluted 1 : 2 in fresh medium after 2¾ hr. and every ½ hr. subsequently.

and then gradually falls towards that of the untreated cells, though even at 5½ hr., after nearly 7 cell generations, it is still about 7 times higher than in the control.

The constant rate of penicillinase production over a period when the cells are increasing their total mass over 100-fold, and are actually growing exponentially, is striking. The Q penicillinase of the treated cells does, however, eventually return to the normal basal level on continued incubation, as would be expected. An 18-hr. culture of penicillin-treated cells in broth, using a small inoculum, resulted in complete loss of all the adaptation originally developed, and no "genetically permanent" change has yet been found to follow the original penicillin treatment.

DISCUSSION.

The general characteristics of penicillinase production by *B. cereus* in the presence of 10,000 units/ml. penicillin are similar to those of other cases of microbial enzyme adaptation. This particular case of adaptation, however, presents two new features not hitherto reported. The first is the pronounced effect of minute concentrations of penicillin (less than 10^{-8} M) in stimulating adaptation; and the second is the acquired ability of cells to form penicillinase in a penicillin-free medium merely by treating them at 0° for a few minutes with small amounts of penicillin, followed by its removal. It is not improbable that the first phenomenon follows inevitably from the second.

It is, of course, uncertain how far the case of penicillinase adaptation may be unique—a possibility made, perhaps, more likely because of peculiarities associated with the marked antibacterial action of the substrate. It is certainly different from nitratase adaptation in *Bact. coli*, where it has already been shown conclusively that cells previously adapted to (i.e. treated with) nitrate do not form the enzyme, when incubated in a nitrate-free medium, any more rapidly than control untreated cells (Wainwright and Pollock, 1949). On the other hand, Wainwright (1950), in further studies on the effect of substrate concentration on nitratase adaptation, has found that a low concentration of nitrate (10^{-4} M) is sufficient to cause half-maximal adaptation when added directly to the medium. It is possible that similar results will be found in other systems. In any case, the fact that such small concentrations of substrate can promote rapid adaptation, even in one or two instances, should be a warning against ascribing activity in promoting adaptation to substrate analogues, etc., which are not of a very high degree of purity. In particular, it throws doubt on the significance of previous reports that the products of enzyme action are often as active in stimulating adaptation as the substrate itself (Yudkin, 1938). In the case of penicillinase, penicilloic acid, when eventually freed from contaminating traces of penicillin, was quite inactive; while as little as 0·001 per cent penicillin impurity was enough to give almost maximal adaptation.

Whether or no the case of penicillinase is unique, the continued production of the enzyme in the absence of penicillin by penicillin-treated cells is a striking phenomenon. Though not " heritable " in the strict genetic sense, the increased penicillinase content of adapted cells is a character acquired by contact with a specific factor in the environment, and has been shown to persist—in some degree—for at least 7 cell generations. That is genetically a very short time, but biochemically a long time, since it involves over a 100-fold increase in cell protoplasm.

The nature of the specific interaction between penicillin and cells which endows them with the new property of rapid penicillinase formation is being further studied, and will be discussed in greater detail in a subsequent publication. The two salient features of the phenomenon are: (*a*) linear formation of enzyme—even during logarithmic growth of cell mass—which continues apparently indefinitely as long as the cells are maintained in full activity by replenishment with glucose and amino-acids; and (*b*) the relationship between rate of enzyme formation and concentration of penicillin used for pretreatment, with maximal effect at 1 unit/ml. As previously pointed out, conclusions based on curves of enzyme formation must always be qualified by the reservation that the only method of measuring the amount of enzyme is by assaying its activity, and that

therefore it is at least theoretically possible to obtain misleading results due to the presence of enzyme inhibitors or activators. Nevertheless, apart from all the other factors which cannot be discussed here, it would be a most remarkable coincidence if the linear phase of penicillinase formation, as illustrated, for example, in Fig. 9, were simply an artefact. Assuming, therefore, that enzyme activity represents—i.e. is always directly proportional to—enzyme content, the two facts emphasized above suggest that the result of penicillin interaction with the cells may be the formation of a new compound or complex, stable enough to resist decomposition during subsequent cell metabolism and growth, which acts as some sort of specific catalyst stimulating the formation of penicillinase. The amount of this hypothetical catalyst formed would, of course, depend upon the concentration of penicillin present during pretreatment, and would determine the rate at which penicillinase was formed subsequently.

Three further points are worth mentioning :

(*a*) The relationship between penicillin concentration used in pretreatment and subsequent rate of enzyme formation, and in particular the low penicillin concentration needed for maximal effect, suggests that there may be some specific receptor substance within the cells with a high combining affinity for penicillin, which becomes " saturated " at a concentration of 1 unit/ml.

(*b*) Penicillin has, in fact, been shown to be specifically fixed by sensitive staphylococci, very rapidly and under conditions (i.e. at very low concentrations and at 0°) similar to those used in this work (Maass and Johnson, 1949 ; Rowley, Cooper, Roberts and Lester Smith, 1950). However, these penicillin-fixation experiments were done with radioactive (S_{35} marked) penicillin, and do not show to what extent absorbed penicillin remains unchanged within the cell.

(*c*) It seems likely that if absorbed penicillin is directly active in promoting adaptation by virtue of its specific molecular integrity, it must be fixed or protected in some way, such that it is not destroyed by the ever-increasing amounts of penicillinase present.

Whatever the mechanism of this " pretreatment effect," it must be regarded as established that penicillinase can be formed *adaptively* (that is, specifically in response to treatment with the substrate) in the absence of free penicillin. That is an important conclusion, since it disposes of any hypothesis, such as that advocated by Monod (1949) to explain enzyme adaptation, which ascribes to the substrate the role merely of stabilizer of enzyme formed as rapidly in its absence as in its presence. It shows also that it is not necessary (as first proposed by Dubos, 1940) for the substrate to be continuously metabolized in order for adaptation to occur.

SUMMARY.

(1) The adaptive production of penicillinase by washed suspensions of *B. cereus* has been followed by manometric assay of penicillin hydrolysis in a CO_2-bicarbonate buffer.

(2) In the presence of 10,000 units/ml. penicillin but without other source of N and in the absence of cell growth, penicillinase production at 35° follows the usual type of " logarithmic " curve characteristic of other enzyme adaptation processes, and may increase 40-fold over control cells, similarly treated but without penicillin.

(3) This adaptation is stimulated by glucose, O_2 and added amino-acids, and inhibited by oxine.

(4) Very small concentrations of penicillin, down to 0·004 units/ml. (8×10^{-9} M), are enough to cause marked adaptation.

(5) Cells treated at 0° with penicillin and then thoroughly washed will, on subsequent incubation in a penicillin-free medium, form penicillinase at a rate up to 30 times greater than untreated cells. This pretreatment effect occurs very rapidly—being about 50 per cent complete after only 10 minutes—and results in linear production of enzyme during subsequent logarithmic growth in a penicillin-free medium, the "Q penicillinase" value thus returning slowly to normal over a period covering several cell generations.

(6) The rate of penicillinase production increases with increase in the concentration of penicillin used for pretreatment up to 1 unit/ml., at which concentration maximal effect occurs.

I wish to express my thanks to Dr. R. D. Housewright for sending me a subculture of *B. cereus* NRRL B-569 used in these experiments; and to Mrs. E. D. Manson and Miss D. Towler for technical assistance.

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INVESTIGATION INTO THE PRODUCTION OF BACTERIOSTATIC SUBSTANCES BY FUNGI. PRELIMINARY EXAMINATION OF THE TENTH 100 SPECIES, ALL BASIDIOMYCETES, AND REVIEW OF FIRST 500 BASIDIOMYCETES.

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THIS paper, though the tenth in the series under the above title, is the sixth dealing exclusively with Basidiomycetes, on which group work in this Laboratory has recently been concentrated. The work differs slightly from that of previous papers in that all the fungi tested were isolated by ourselves and the method of testing was by the " Wilkins-Revised " method (Wilkins, 1949). Metabolism solutions of the fungi were tested for antibacterial activity against *Bacterium coli* and *Staphylococcus aureus*, and the results arranged in " strongly positive " (SP), " weakly positive " (WP) and " negative " (N) lists as in previous papers. Fungi listed as negative were those which failed to give a positive reaction either in the preliminary Strip test or in the final Hole test, and only the SP species are dealt with in detail.

LIST OF FUNGI EXAMINED.

Strongly Positive.

	Result of test on liquid medium.			
			Zone dia. (mm.).	
	Time in days.	Medium.*	*Bact. coli.*	*Staph. aureus.*
Coprinus picaceus (Bull.) Fr. W616A . .	30	M	—	23
C. picaceus (Bull.) Fr. W616B . . .	30	M	—	26
Crucibulum vulgare Tul. W638A . . .	40	M	—	22
Cyathus striatus (Huds.) Pers. W639A . .	35	M	13	23
Hygrophorus pratensis (Pers.) Fr. W618A .	15	M	—	21/25
Marasmius conigenus (Pers.) Karst. W619B .	20	PD	11	30
M. peronatus (Bolt.) Fr. W613A . . .	35	PD	15	20
Pholiota aegerita (Porta.) Fr. W642A . .	15	M	15	20
Polyporus betulinus (Bull.) Fr. W620B . .	25	M	16	20

* M = malt; PD = potato dextrose.

Weakly Positive.

Clitocybe clavipes (Pers.) Fr. W646A; *C. gilva* Fr. W693A; *Collybia fusipes* (Bull.) Berk. W662A; *Marasmius peronatus* (Bolt.) Fr. W613B; *Poly-*

porus betulinus (Bull.) Fr. W620A ; *Psalliota arvensis* (Schaeff.) Fr. W617G ; *Sparassis crispa* (Wulf.) Fr. W651A ; *Stereum hirsutum* (Willd.) Fr. W6112A ; *Tricholoma nudum* (Bull.) Fr. W61G.

Negative.

Anellaria separata (Linn.) Karst. W6115A ; *Armillaria mellea* (Vahl.) Fr. W688A and W688B ; *A. mucida* (Schrad.) Fr. W643B ; *A. robusta* (A. & S.) Fr. W699A ; *Clitocybe cyathiformis* (Bull.) Fr. W621A, W621B and W621C ; *C. nebularis* (Batsch.) Fr. W644B ; *C. vibecina* Fr. sec Qúel. W65A ; *Collybia butyracea* (Bull.) Fr. W610A and W610B ; *C. fusipes* (Bull.) Berk. W662B ; *C. longipes* var. *badia* Lucand. W692A ; *C. radicata* (Rehl.) Berk. W69A and W69B ; *C. velutipes* (Curt.) Fr. W6111A ; *Coprinus atramentarius* (Bull.) Fr. W640A ; *C. comatus* (Fl. Dan.) Fr. W623A and W623B ; *C. micaceus* (Bull.) Fr. W6110A ; *Cortinarius* (*Derm.*) *anthracinus* Fr. W686A ; *Fistulina hepatica* (Huds.) Fr. W632A ; *Flammula sapinea* Fr. W624B and W624C ; *Fomes annosus* Fr. W698A ; *Ganoderma applanatum* (Pers.) Pat. W6103A ; *Hypholoma fasciculare* (Huds.) Fr. W635A and W635B ; *H. hydrophilum* (Bull.) Fr. W697A ; *Inocybe geophylla* (Sow.) Fr. var. *lilacina* Fr. W654A ; *Lepiota procera* (Scop.) Fr. W625A ; *Lycoperdon perlatum* Pers. W695A ; *Marasmius alliaceus* (Jacq.) Fr. W67A ; *M. dryophilus* (Bull.) Karst. W636A ; *M. erythropus* (Pers.) Fr. W687A ; *M. hariolorum* (D.C.) Qúel. W683A ; *M. oreades* (Bolt.) Fr. W655A ; *Mycena epipterygia* (Scop.) Fr. W612A and W612B ; *M. galericulata* (Scop.) Fr. W626A ; *M. polygramma* (Bull.) Fr. W611A and W611B ; *Naucoria pediades* Fr. W663A ; *Panaeolus sphinctrinus* Fr. W630A ; *Panus stipticus* (Bull.) Fr. W647A ; *Phallus impudicus* (Linn.) Pers. W657A ; *Phlebia merismoides* Fr. W6107A ; *Pholiota aurivella* (Batsch.) Fr. W658A ; *Pluteus cervinus* (Schaeff.) Br. W660A ; *P. salicinus* (Pers.) Fr. W610A ; *Polyporus adustus* (Willd.) Fr. W629A ; *P. betulinus* (Bull.) Fr. W620C ; *P. sulphureus* (Bull.) Fr. W634A ; *Polystictus versicolor* (Linn.) Fr. W627A, W627B, W627C and W627D ; *Psalliota augusta* Fr. W622A ; *P. arvensis* (Schaeff.) Fr. W617A, W617B, W617C, W617K, W617L, W617M, W617N, W617P, W617T, W617U and W617V ; *P. comtula* Fr. W631A and W631B ; *P. hortensis* (Cke.) W. G. Sm. W68A ; *Sparassis laminosa* Fr. W6100A ; *Stereum sanguinolentum* (S. & S.) Fr. W6113A ; *Stropharia aeruginosa* (Curt.) Fr. W628B and W628C ; *Trametes gibbosa* (Pers.) Fr. W66A ; *Tricholoma nudum* (Bull.) Fr. W61D ; *T. personatum* Fr. W62B ; *T. rutilans* (Schaeff.) Fr. W696A ; *Volvaria bombycina* (Schaeff.) Fr. W64A.

In general, the above results show no marked differences from those of previous papers.

Review of Work on the 500 Species of Basidiomycetes.

Excluding the fungi in the present paper, I have examined 500 Basidiomycetes, representing 422 species from 65 genera, and have tested them for antibacterial activity by the original Wilkins method (Wilkins and Harris, 1943). The cultures were obtained from three different sources, the source being indicated in the lists by the method of numbering, e.g., from the Centraalbureau voor Schimmelcultures, Baarn, Holland, no number ; from the Forest Products Research Laboratory, Princes Risborough, a number after the name ;

from Professor Robbins, New York Botanic Gardens, a number, a letter or both in a bracket after the name.

It seems that any review of results of this investigation of 500 spp. of Basidiomycetes might very conveniently include reference to similar investigations by other workers. The only important comparable work is that done by Robbins, Hervey, Davidson, Ma and Robbins (1945), who examined 300 Basidiomycetes, and with whose paper comparisons have already been made (Wilkins, 1947*b*), and more recently Hervey (1947), who has examined 500 spp. (actually 511), and whose results will briefly be compared with ours. Whereas Robbins and I exchanged cultures and compared notes, the examination by Hervey was completely independent of mine. Direct comparison with Hervey is of necessity approximate because of the different methods of growing and testing. The media on which the fungi were grown were essentially different, and for testing we used the Hole method with metabolism solution (Wilkins and Harris, 1943), whereas Hervey, in general, used the Streak method on plates.

Our classification of results into SP, WP and N mentioned before is based on a proportion of the maximum zone of inhibition expected, SP being those spp. which give a zone width of 50 per cent or more of the expected maximum of 10 mm., i.e., a zone width of 5 mm. or over, and WP those with a zone width of 2·5 to 5 mm. Anything below that is counted as negative, and these are added to those that were negative in the initial Strip test. Hervey's results can be evaluated on the same proportional basis. Table I shows the comparative distribution of SP, WP and N results by Wilkins and Hervey.

TABLE I.—*Comparison of Positive and Negative Results, Wilkins and Hervey.*

	Genera.			Species.			
	Total.	Positive.	Negative.	Total.	Positive. Strong.	Weak.	Negative.
Wilkins	65	43	22	500	79	155	266
					234		
Hervey	64	47	17	511	83	121	307
					204		

Table I shows that in spite of the different fungi examined and the different testing methods, the results are remarkably similar, and in both cases the number of positives was approximately 50 per cent of the total, and the number of SP spp. was approximately 30 per cent that of the positives, i.e., approximately 15 per cent of the total. This tends to confirm our idea that in any such examination of Basidiomycetes by any observer and tested by any method, the results will be approximately 15 per cent SP, 35 per cent WP and 50 per cent N.

Comparison of the genera shows close agreement. In this case "positive" means that at any rate some species in the genus were positive. Out of a total of 65 genera examined by Wilkins, 43 had some positive spp., while out of Hervey's total of 64 genera, 47 were positive. In the negative genera 22 and 17 respectively of the genera of each author were represented by only one spp. so that deductions as to the antibacterial activity of these genera can have but little significance. Though unwilling to make definite statements, we are inclined to regard certain genera as having few or no positive spp., while other genera

produce a relatively high proportion of positive spp. Fifty-two genera were common to both authors, and of the six genera with the highest number of isolates, five genera are common to both. Table II shows the comparative results of these five genera.

TABLE II.—*Comparison of the Distribution of Antibacterial Activity of Five Genera by Wilkins (W.) and Hervey (H.).*

Genera.	Observer.	No. of isolations.	Results.		
			SP	WP	N
Coprinus	(W.)	24	7	5	12
	(H.)	39	2	3	34
Fomes	(W.)	65	7	15	43
	(H.)	40	4	16	20
Polyporus	(W.)	72	10	25	37
	(H.)	95	11	33	51
Poria	(W.)	28	7	14	7
	(H.)	80	13	29	38
Trametes	(W.)	21	2	9	10
	(H.)	29	9	13	7

Table II shows the variable degree of agreement very well. In *Fomes* and *Polyporus* agreement is relatively close, but *Coprinus*, which has the highest percentage of SP isolations according to Wilkins, has the lowest percentage according to Hervey, while the converse is true of *Trametes*.

Agreement is less emphasized when results of the species tested by both Wilkins and Hervey are compared. Of the 266 spp. common to both, 40 per cent of the results were not in agreement. Of the 117 spp. recorded as N by Wilkins, Hervey records 29 P out of which 8 were SP, and out of the 110 recorded N spp. of Hervey, Wilkins finds 23 P of which 9 are SP. The fact that an authentic species can be regarded as negative by one person and strongly positive by another confirms the opinion that, in general, a species can rarely be described as either positive or negative. The antibacterial activity is, in fact, a function of the individual rather than the species, and even when the same isolates are tested by different people, results often vary. Both authors, however, recorded as SP the species given below, and these can therefore be regarded as reasonably dependable SP:

Coprinus picaceus.
C. plicatilis.
Fomes annosus.
F. juniperinus.
F. officinalis.
Lenzites heteromorpha.
Merulius lachrymans.
Polyporus betulinus.
Poria xantha.
Trametes serialis.

The nett result of this examination of 500 spp. can be summarized as follows: The consistently SP fungi belong to fungi of the wood-destroying type, though on the other hand many wood-destroying fungi are invariably negative.

Few species show consistent antibacterial activity; there is usually a wide range of strain variation in any species.

Certain genera, e.g., *Fomes* and *Clitocybe*, produce a relatively high proportion of SP spp., while others, e.g., *Armillaria*, *Collybia* and *Mycena*, are characteristically negative.

Little is known of what causes a fungus to be positive, or why a positive fungus should, quite suddenly, cease to give positive results.

The medium on which the fungus is grown is a determining factor in relation to its antibacterial activity.

Different testing methods lead to different results.

The " Hole " method of testing indicates the degree of antibacterial activity only within limits; the ultimate test should be of the " serial dilution " type.

There is, in our opinion, no direct relation between rate of growth of a fungus and the degree of its antibacterial activity.

I am indebted to the Medical Research Council for a grant enabling me to employ Research Assistants for this work, and in this connection I am grateful for valuable help from Miss B. M. Partridge, B.A. (whose name appears as joint author), Miss B. Knee, B.A., Miss J. Rose and Mrs. S. D. Edwards.

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THE HISTOLOGY OF "GREY LUNG VIRUS" LESIONS IN MICE AND COTTON-RATS.

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THE biological and physical characters of grey lung virus, a pneumotropic agent affecting mice, were briefly described by Andrewes and Glover (1945), and some account was given of the histopathology of the lesions in mouse lung. The virus has most unusual properties in that both it and the lesions it produces persist apparently indefinitely in infected animals. Though the virus is still present in large amounts and no immunity is demonstrable, the lesions remain almost static for many months and rarely lead to death. These facts, together with a later finding that grey lung virus is sensitive to some antibiotics, suggest that it may belong to a distinct virus group and call for a more detailed description of the histogenesis of the lung changes. This is all the more necessary as similar agents have been discovered by Dr. K. Goodner (1950, personal communication) in mouse stocks in Philadelphia ("Potash Virus"), in cotton rats by Dr. H. Rose (1950, personal communication) in New York, and also in normal cotton rats in this Institute (Andrewes and Niven, 1950).

METHODS.

Grey lung virus produces pathological changes only in the lungs, and in the more recent studies in mice and cotton rats, examination was limited to these organs.

The thoracic contents were removed *in toto*, the heart usually excised and the remainder immersed in fixative. The containers were placed immediately in a desiccator in which a partial vacuum (60 mm.) was obtained by means of a water pump. It is important not to increase the vacuum beyond this level, and when evacuation of air from the lungs is complete, air must be allowed to re-enter the desiccator slowly. In most instances, corrosive sublimate containing 4 per cent glacial acetic acid was used as the fixative: Bouin's fluid, Zenker-formol, Zenker's fluid + 4 per cent glacial acetic acid and formol-saline were occasionally employed. After the air had been exhausted, an oblique cut was made through both lungs and the tracheo-bronchial lymphatic glands with the object of having a plane of section through as many lobes and structures as possible. In a few cases, the skin was removed from the thorax and the whole animal immersed in formol-saline for four days (M. Straub, 1945, personal communication) after which the lungs were cut out and fixation was continued in corrosive sublimate alone for 24 hours. This allowed an accurate appreciation of the amount of fluid and air respectively in the lungs, but is, of course, an unsuitable technique for general histological purposes. Many staining methods have been employed—

haematoxylin and eosin, Mann's stain, phloxine-methylene blue, Giemsa's mixture, orcein for elastic tissue and various trichrome methods for collagen and reticulum fibres and a coelestin blue technique (W. Penny, 1948, personal communication) for mucin.

RESULTS.

The disease in mice: Histogenesis.

The interalveolar septa, 48 hours after inoculation, are more conspicuous owing partly to capillary congestion and also to enlargement of the septal cells. When the lungs are examined in their entirety, a few small areas of consolidation involving only two or three adjacent alveolar sacs can always be found. These tiny foci consist almost entirely of polymorphonuclear leucocytes, with only a small admixture of desquamated, slightly enlarged alveolar septal cells, and occur most commonly below the pleural surface, although they are occasionally seen more deeply in the lung substance close to the hilum (Fig. 1). After a further 48 hours, the prominence of the alveolar septa is yet more pronounced; they contain an excess of polymorphs, and the septal cells are not only enlarged and projecting into the alveolar spaces but are increasing slightly in numbers. The foci of consolidation have increased in number and size, and may be just visible macroscopically. By the sixth day, the areas of consolidation have enlarged considerably and are easily visible (Fig. 2). Although polymorphs still predominate, many septal cells now lie free amongst them in the alveolar spaces. At this stage the cells have an oval or round vesicular nucleus and fairly abundant sharply-defined eosinophilic cytoplasm; mitotic figures are quite numerous both *in situ* on the septal walls and in the alveolar spaces; phagocytic activity is also evident, polymorphs in all stages of disintegration being found within them. Almost all the alveolar spaces, whether or not they contain cells, show fluid exudate, and perivascular oedema of medium-sized and small arterioles is also apparent. Until this stage, no change has been found in the epithelial lining of the respiratory tract, but from the sixth to eighth day onwards, a slight

EXPLANATION OF PLATES.

FIG. 1.—Primary focus of intra-alveolar consolidation composed of polymorphonuclear leucocytes and desquamated septal cells: 48 hours after infection. × 180.

FIG. 2.—Six days after infection: large area of consolidation consisting of polymorphs and small numbers of septal cells. Bronchioles and alveolar ducts are patent. × 70.

FIG. 3.—Enlargement and detachment of alveolar septal cells which stain intensely and have sharply defined outlines: 8 days after infection.

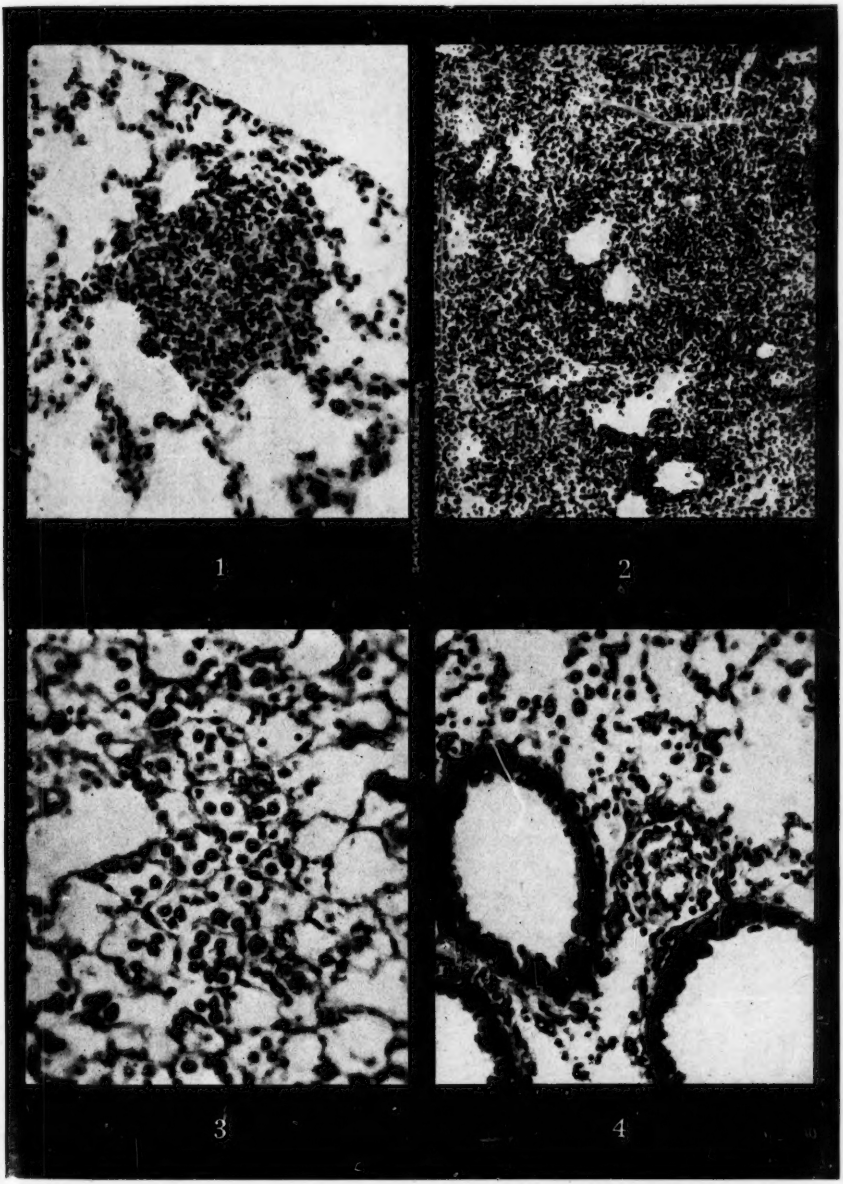
FIG. 4.—Hyperplasia of bronchiolar epithelium: an arteriole in the centre of the field shows marked perivascular oedema and a few infiltrating lymphocytes: 8 days after infection. ×245.

FIG. 5.—One month after infection: bronchi, bronchioles and alveolar ducts remain patent; alveolar sacs contain fluid exudate and varying numbers of cells. Mononuclear cells surround bronchi and blood vessels. × 55.

FIG. 6.—Six months after infection: dilated lymphatic channels amongst the plasma cells surrounding blood vessels. × 70.

FIG. 7.—Four months after infection: greatly enlarged and vacuolated alveolar cells. The interalveolar septa show increased cellularity. × 245.

FIG. 8.—Ten months after infection: the spaces in the lung substance have been formed by the disintegration of intra-alveolar cells and related septa. × 245.



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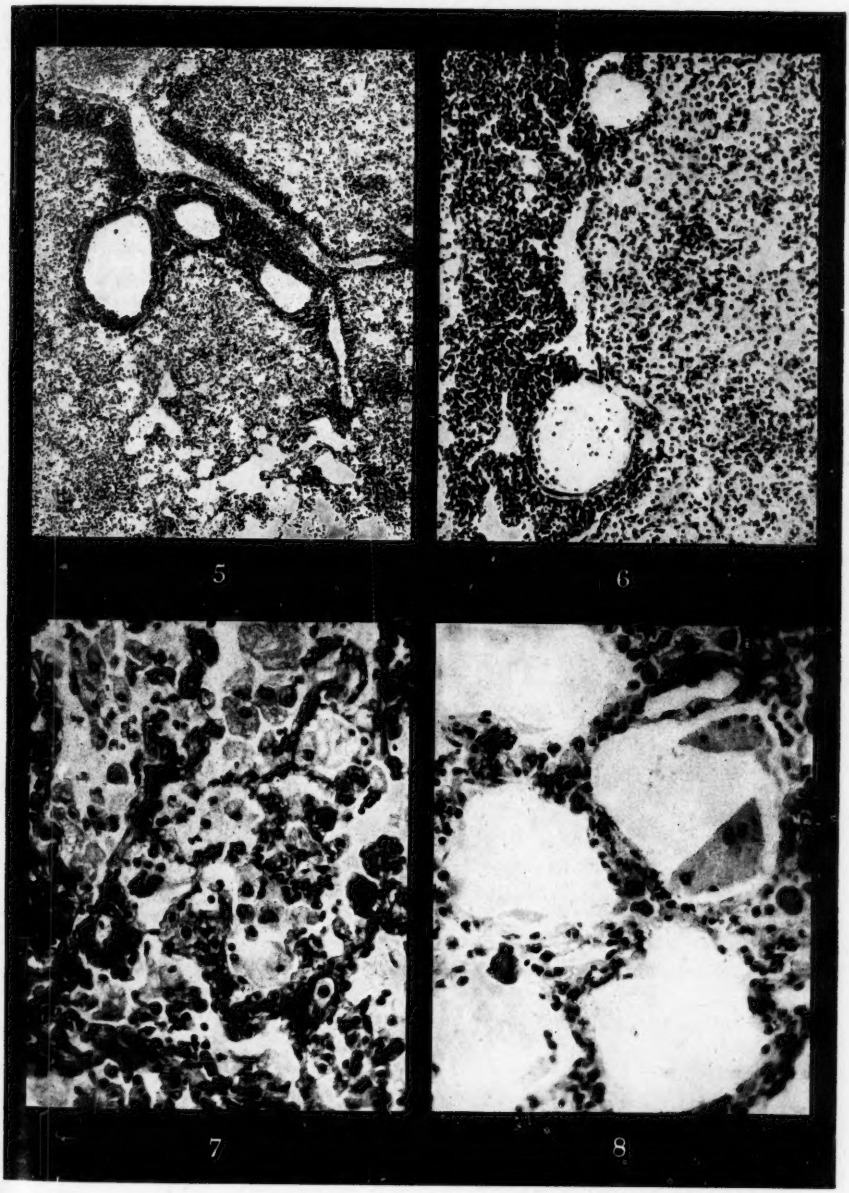

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but definite increased cellularity of medium-sized and smaller bronchi is obvious, mitotic figures being readily found. The newly-formed cells are packed closely together on the basement membrane, and the hyperplasia does not produce any fundamental alteration of the epithelium (Fig. 4). It must be emphasized at this point that these tissue alterations are not identical in extent either in each lung or in all the animals of a series inoculated at the same time, nor is there any localization in any particular lobe although the small azygos lobe does seem to be consistently involved at an early stage. Moreover, the rate of evolution of the lesions varies considerably ; at the eighth day, when three out of six animals are showing considerable consolidation, the remainder may have only small subpleural foci, interstitial infiltration of polymorphs and early mobilization of the septal cells. The extent of the polymorph reaction is also another variable feature ; it is primarily focal, and is the most conspicuous element of the initial reaction to inoculation, but mobilization of the septal cells is not everywhere in the lung preceded by an outpouring of polymorphs (Fig. 3). By the tenth day, a loose infiltration of mononuclear cells of lymphocyte type surrounds many of the arterioles and obscures the perivascular oedema. Many of the polymorphs have disappeared, partly by ingestion and partly by way of the lymphatics, degenerate forms being readily found in the sinuses and within the reticulo-endothelial cells of the tracheo-bronchial lymphatic glands. By the twelfth day, hyperplasia of the mesothelial lining of the pleura is evident, at first focal in relation to well-developed alveolar lesions and later generalized. Lymphocytes now occur around the bronchi and bronchioles, and the epithelial hyperplasia observed at an early stage is now evident throughout the entire bronchial tree.

Intra-alveolar oedema becomes generalized, and the subsequent evolution of the lung lesions is brought about by changes in the various cellular elements which have appeared during the first twelve days in response to the introduction of the causative agent. Macroscopically, the greyish colour of the lungs, the enlargement and the absence of collapse when the thorax is opened are the most striking findings observed. Although the cells in the alveolar sacs and the areas of round cell infiltration contribute to the enlargement, the most important factor is the exudate, for as fluid oozes out of the pulmonary tissue when it is incised, a marked diminution in the size of the lungs occurs. Hepatization, such as is found in a primary bacterial pneumonia, never takes place, except in late stages and in focal areas following secondary bacterial invasion. The small emphysematous patches which can sometimes be recognized macroscopically are either dilated alveolar ducts which always remain patent or groups of dilated alveoli whose walls remain normal or show only moderate swelling of the septal cells *in situ*.

The fundamental pattern of the disease is now established, and it is convenient to enumerate and describe separately the essential components. The polymorphonuclear leucocytes, so conspicuous in the first ten days following inoculation, do not come into that category ; they represent an immediate reaction to the introduction of the causative agent and have disappeared as a rule within two weeks after inoculation (Fig. 5). At a later stage, when degenerative changes or secondary infection occur, they may reappear in considerable numbers. The large mononuclear cells in the alveoli, the fluid exudate, the epithelial and mesothelial hyperplasia and the perivascular and peribronchial

infiltration, on the other hand, form permanent features and persist although not necessarily unchanged throughout the course of the disease.

Alveolar cells.

Following disappearance of the polymorphs, the affected alveoli come to contain increasing numbers of cells derived from the alveolar walls. They originate from the " septal cells " ; this is a useful non-committal term for the inconspicuous cells located in the intercapillary spaces, and has the advantage of not implying either a mesenchymal or epithelial origin. Their enlargement *in situ* and separation from the septa can be readily seen, and they increase in numbers by mitotic division ; bi-nucleate forms are also frequently found. In the early stages of the condition (Fig. 3) up to about two months after infection, they have an intensely eosinophilic cytoplasm, but gradually vacuoles appear in increasing numbers, the cytoplasmic outlines become less sharply defined and the nucleus shrunken (Fig. 7). At a later stage, lipoid substances are deposited in the cells, sometimes in the form of doubly refractile rhomboidal plates, but this fatty change is a variable feature both in time of appearance and in extent. It has to be noted also that after the first two months, the appearance of the intra-alveolar cells varies in different parts of the lung ; in some areas considerable vacuolation may be present, while in others, they may still show a homogeneous eosinophilic cytoplasm and vesicular nuclei : it seems likely that the latter areas represent recently involved portions of the lung. In the early stages the cells show phagocytic activity and ingest polymorphs ; at a later stage, from about four months onwards, many contain iron pigment derived from phagocytosed erythrocytes. Although there is no fundamental alteration in the basic micro-anatomical structure of the lung, the presence of intra-alveolar exudate and cells must, at least focally, slow down the flow of blood through the capillaries and the occasional identification of small capillary thromboses supports this possibility. Small haemorrhages are then liable to occur, and the extravasated erythrocytes are dealt with in the usual way by the alveolar cells. As time passes, the alveolar cells continue to enlarge, the vacuolation increases, the nuclei become pyknotic and finally, the cells disintegrate. This can occur in individual cells in an alveolus, but more generally small groups of consolidated alveoli are affected simultaneously and the interalveolar septa disappear at the same time (Fig. 8). Polymorphs often invade the broken-down tissue, but quite often large fluid-containing spaces are the only end result of this degenerative process. As already stated, the mobilization of septal cells and their accumulation within the alveolar sacs is never generalized throughout the lungs and it would appear that about the second and third month after infection this reaction slows down and possibly stops. Thus, even at an advanced stage, one can always find areas of lung with an almost normal alveolar structure and showing only a moderate enlargement of septal cells as well as areas where the alveolar sacs contain only a few desquamated cells which still possess an eosinophilic non-vacuolated cytoplasm.

Fluid exudate.

Accumulation in the alveolar spaces of fluid in which fibrin is scanty or absent, is one of the contributory causes to the enlargement of the lungs which is such

a prominent feature of this condition; the abundant fluid which flows from the cut surfaces when the lungs are excised is truly remarkable. The presence of this exudate is probably responsible for the dilatation of the perivascular lymphatics, a constant finding three to four weeks after infection, and for the enlargement of the tracheo-bronchial glands. In the later stages of the disease, the products of alveolar cell disintegration are added to the fluid content of the lungs. Exudation of fluid follows the cellular reaction and seems, during the second month, to reach a maximum level which is maintained throughout the course of the disease.

Perivascular and peribronchial infiltration.

The formation of thick cuffs of mononuclear cells ensheathing the blood vessels and air passages, from their commencement in the lung at the hilum to their ultimate subdivisions at the level of capillary and alveolar duct respectively, plays a large part in determining the characteristic macroscopic appearances of the lungs; the actual bulk of tissue so formed can occupy a very considerable proportion of the total lung volume. Perivascular oedema which begins about the sixth day after infection around the small and medium-sized arterioles, precedes the onset of infiltration, but is soon obscured by increasing numbers of mononuclear cells. At first of small lymphocyte type with scanty cytoplasm and densely-staining nucleus, they soon acquire the characters of the medium-sized lymphocyte and proliferate actively. The infiltration is always more extensive round arteries and arterioles than around the bronchi, bronchioles and veins. Perivascular lymphatics are not obliterated and remain as conspicuous channels with intact endothelial lining (Fig. 6). Although occasionally isolated macrolymphocytes have been recognized in these cellular accumulations, no sharply defined cellular structures have ever been made out. In the later stages, the cells may come to acquire the characteristic morphology and tinctorial qualities of plasma cells and this, when it occurs, is always accompanied by a similar transformation in the tracheo-bronchial lymphatic glands. Although the process is progressive in character, the infiltrating cells do not invade the alveolar spaces, but adjacent alveoli become flattened out against the edge of the cellular areas. Moreover, the increasing bulk of this infiltration outside the respiratory epithelium tends gradually to narrow the lumen of the air passages, particularly of the terminal bronchioles, and may be an indirect factor favouring secondary bacterial invasion. Very occasionally in the terminal bronchioles in the late stages of infection mononuclear cells penetrate the basement membrane in small groups, but although the epithelium becomes flattened over them, ulceration has never been found.

Epithelial hyperplasia.

Most pneumotropic viruses attack directly bronchial and bronchiolar epithelial cells. The mouse pneumonia virus of Nigg (Nigg and Eaton, 1944) multiplies within the cytoplasm of individual cells which become stuffed with elementary bodies and eventually rupture. In experimental influenza in the mouse (Straub, 1937), considerable focal areas of epithelial necrosis, preceded by the appearance of eosinophilic granules in the cell cytoplasm, are produced at any part of the bronchial tree. In grey lung disease, however, no such host-

virus relationship is obvious, and the respiratory epithelium shows only a mild degree of orderly hyperplasia particularly of the ciliated cells; this begins a few days after infection, has no obvious relationship to cell loss and persists throughout the course of the disease. Since the normal arrangement on the basement membrane is maintained, proliferation produces a degree of folding of the epithelium as a whole and the cells are moreover more columnar. In the later stages, goblet cells, usually present in small numbers only in the large bronchi, increase greatly in numbers and abundant mucin is produced.

Mesothelial hyperplasia.

Mesothelial hyperplasia is confined to the visceral pleura, varies very much in degree and may be generalized or focal. Mononuclear cells frequently accumulate in small groups and may give rise to small flat papillomatous projections: this is the only situation within the lung where their occurrence is unrelated to blood vessels or air passages.

Other changes.

Chronic bacterial infection of the lung of long duration is often accompanied by considerable overgrowth of connective tissue leading to permanent alteration of structure. Amongst virus infections, however, only influenza in the mouse produces irreversible changes, considerable areas of lung becoming epithelialized by the ingrowth of metaplastic epithelium from the terminal bronchioles into the alveoli (Straub, 1940). In grey lung infection, although no new formation of connective tissue disturbs the general micro-anatomical plan, the existing collagen and elastic fibrils in the interalveolar septa gradually become thicker and more numerous, particularly in consolidated areas and where the cellular content of the septa is increased, this change being most marked at the junction of alveolar ducts and air sacs. At a still later stage, however, the interalveolar framework within an area of consolidation in which the cells have become greatly enlarged and vacuolated eventually atrophies, and as the cells break down, the fibrils also disintegrate and disappear (Fig. 8). Abundant new formation of reticulum fibres occurs amongst the perivascular and peribronchial cuffs of mononuclear cases. In one case examined a year after infection, a perivascular cuff was replaced by dense hyaline connective tissue in which only a few mononuclear and plasma cells survived.

Secondary infection.

At one time the passage material was contaminated with the Nigg pneumonia virus, and bronchiolar and alveolar cells containing elementary bodies could be found, although never in very large numbers. During this period the primary lesions resembled uncomplicated Nigg pneumonia not only histologically, but also in their rapid development, and it was this similarity which prompted a careful day-to-day study. After treatment with sulphamerazine, the Nigg virus was eliminated and the usual histological course of the condition was re-established, the grey lung virus being quite unaffected by the drug. Gross bacterial infection sometimes occurs at a later stage of the disease, usually from 9 to 15 months after inoculation and the histological picture changes completely. The lungs become overrun with polymorphs, and small abscesses develop in

relation to terminal bronchioles. The larger bronchi are packed with polymorphs, the epithelium undergoes metaplasia and comes to consist of several layers of non-ciliated stratified cells. Around the abscesses and the bronchi considerable new formation of connective tissue takes place.

The disease in cotton rats.

Grey lung disease can be transmitted to cotton rats (Andrewes and Glover, 1945) and the infection follows the same course. Histologically a somewhat similar picture is produced but fluid exudate is scanty, pleural changes are slight and in the first series examined, the mobilized septal cells did not show the same tendency to vacuolation and disintegration nor were the mononuclear cell infiltrations so extensive as in the mouse. In a subsequent series, however, although fluid exudate remained minimal, the alveolar cells underwent similar cytological changes, and perivascular and peribronchial infiltrations were just as widespread as in the mouse. Epithelial hyperplasia, thickening of the framework of the interalveolar septa, prominence of lymphatic channels were also present. On histological grounds alone, it was found impossible to distinguish between grey lung disease in cotton rats and lesions in cotton rats produced by a virus recently isolated in that species (Andrewes and Niven, 1950).

DISCUSSION AND SUMMARY.

Differentiation of the grey lung agent from other pneumotropic viruses which have probably originated in mouse stocks, and from murine pleuropneumonia-like organisms was discussed by Andrewes and Glover (1945), and it is sufficient to recapitulate their main points. The absence of elementary bodies and lethal action distinguishes it from viruses of the Nigg pneumonitis group ; failure to provoke immunity, absence of antibodies and low pathogenicity differentiate it from the pneumonia virus of mice (P.V.M.) of Horsfall and Hahn (1940). An agent of the pleuropneumonia group cannot be excluded so definitely, but no new evidence has become available to support such a view. Histologically, grey lung virus behaves quite differently in mouse lung from the Nigg virus which causes lesions always related to the multiplication of elementary bodies within susceptible cells. In the case of P.V.M., histological differentiation is much less certain. Horsfall and Hahn describe fluid exudate and mononuclear cells in the alveolar spaces, an increased cellularity of the interalveolar septa and abundant perivascular and peribronchial infiltration. As already stated, however, the serological response in this disease is quite specific, and moreover, a haemagglutinating agent for murine erythrocytes can be extracted from infected lungs (Mills and Dochez, 1944). Infected animals succumb in 10 to 14 days following inoculation of material which has undergone a few mouse passages and resemblance is confined to the actual lung changes. Although the pulmonary tissue reactions to grey lung virus are thus not specific, it is unique in maintaining pathological changes apparently indefinitely. On histological grounds alone, it is impossible to decide which of these are due primarily to the virus and which are non-specific. Perivascular and peribronchial infiltration occur in response to many agents both in lethal and sublethal doses and belong to the latter category. The rapid disappearance of the alveolar cells

and repression of the epithelial hyperplasia following antibiotic therapy (Andrewes and Niven, 1950) do suggest, however, a close association with the causative agent. The oedema also disappears very quickly, and it cannot be denied that the virus may alter capillary permeability, the occasional haemorrhages being the result of a further degree of capillary dysfunction and not due to mechanical factors as suggested earlier. The chemotherapeutic experiments also show clearly that the alterations of lung structure unlike those sometimes produced by influenza virus (Straub, 1940) are not irreversible. The possibility that grey lung virus is an agent with neoplastic potentialities has also been considered, and with this in mind lungs from many animals 12 to 18 months after infection have been examined, without, however, finding any evidence of uncontrolled proliferation in the epithelium lining the bronchial tree, the most likely site of neoplasia. In two instances, both in animals inoculated 18 months previously, a single focus of fibroblastic proliferation, unrelated to secondary infection which was present elsewhere, was found near the tip of a lobe and close to the pleural surface; in neither case were mitoses present, and the lesions could not be regarded as new growths within the framework of the lung.

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CHEMOTHERAPEUTIC EXPERIMENTS WITH GREY LUNG VIRUS.

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GREY lung virus, described by Andrewes and Glover (1945), is an agent pathogenic for mice, cotton-rats and, to a lesser extent, other rodents. Its natural habitat is uncertain; though possibly of rodent origin it does not now exist as a latent infection of our laboratory mice. While many viruses persist for long in recovered animals, grey lung virus exhibits this property *par excellence*: it rarely kills its host, but causes extensive lung lesions which remain indefinitely—at least for eighteen months. Virus, also, persists indefinitely in the lungs and in high titre: neither active immunity nor antibody-production have been demonstrable.

While on a visit to Dr. K. Goodner, of Jefferson Hospital, Philadelphia, in October, 1949, one of us (C. H. A.) was shown a rather similar condition in mice under study there, and was informed that that infection ("Potash virus") was susceptible to treatment with aureomycin. Accordingly, on returning to England tests were made of the activity of aureomycin against grey lung virus.

EXPERIMENTAL METHODS.

Mice were inoculated intranasally under ether with centrifuged suspensions of infected lungs (0·05 ml.); these were either freshly obtained from mice killed at any time from a week to a year after infection, or were removed from storage in a dry ice (— 76° C.) container. The experimental mice were killed 9 to 13 (usually 10) days after infection and an assessment made of the extent of lung-involvement. In untreated animals this was usually so extensive as to involve all lobes, which were distended, grey and oedematous. So few mice died that mortality was useless as a measure of the effectiveness of treatment. The dosage of virus used was of the order of 10^4 to 10^5 m.i.d.

Aureomycin was suspended in physiological saline containing 5 per cent gum acacia. In most experiments the strength of aureomycin was 2 mg./ml., and 0·5 ml. was given subcutaneously. Such injections produced moderately severe local reactions, so the site of injection was varied at each dose.

RESULTS.

Effect of Aureomycin.

When mice were given 1 mg. aureomycin once or twice daily from the day of inoculation until the tenth day afterwards, lung lesions were wholly suppressed (mice killed on thirteenth day).

The histological structure of the lungs of treated mice was always compared with that of untreated animals inoculated at the same time. The development

TABLE I.—*Effect of Aureomycin on Grey Lung Virus.*

Lesions in		
Control mice.	Mice on 1 daily dose.	Mice on 2 daily doses.
+++ +++ +++	0 0 0 0	0 0 0 0
+++ +++ +++	0 0 0	0 0 n.sp.*

+++, ++, +, 0 indicate degrees of lung involvement.
n.sp. = non-specific lesion: no virus recovered on passage.
* = non-specific death.

of the lesions up to the end of the usual experimental period (10 to 14 days) has already been described (Niven, 1950). At this stage abundant fluid exudate is already present in the alveolar spaces, and considerable areas of consolidation, composed of enlarged desquamated septal cells and varying numbers of polymorphs have formed. Infiltration of mononuclear cells around arteries, and to a lesser extent around bronchioles and bronchi, has also begun, and an increased cellularity of the epithelium of the respiratory tract generally within the lung is apparent.

Following treatment for 10 days, histological examination confirmed the impression gained from naked-eye inspection that the lungs in the treated animals were normal. No cellular accumulations or fluid exudate were found in the alveolar spaces, and the walls of the blood vessels and bronchi were free from infiltrating cells. Only occasionally in the septal capillaries was a slight excess of polymorphs noted. That the causative agent did in fact obtain a foothold in the lungs for a short time before its activity was cut short by aureomycin administration is suggested by the abundance of polymorphs in the sinuses and within phagocytes in the tracheo-bronchial glands.

The suppressive effect of aureomycin was reproduced in many subsequent experiments, but such drastic treatment was not found necessary. Almost complete suppression of lesions was produced by daily doses of 0·25 mg., but the results were less consistent. Only in three out of nine animals of the series were the lungs identical on histological examination with those of the previous series (1·0 mg. per day). In the others, lesions varying from small sub-pleural areas of consolidation to almost complete lobar involvement were found, but the amount of fluid exudate was always much less or absent, polymorphs were much less numerous, and mononuclear infiltration less extensive. Since one daily dose was effective, this routine was used in all later experiments. Good results were obtained and lesions apparently wholly suppressed when beginning of treatment was delayed until five days after infection. Microscopic examination showed, however, that suppression of the lesions was not quite complete. In addition to a few small areas of perivascular infiltration, the capillaries generally showed an excess of polymorphs, and at the periphery of the lung, where areas of consolidation most commonly first appear, the alveolar septa were enlarged and prominent. The alveolar spaces, however, contained neither fluid nor free cells. In each of two tests lesions were suppressed by only two doses of 1 mg., given at the time of inoculation and on the following morning. In one experiment a single dose of 1 mg. given at the time of infection was equally successful, but another similar attempt, against more potent virus, failed. Administration of drug into the stomach, by the aid of a syringe fitted with a wide blunted needle,

was also effective, but larger doses (2·5 to 5 mg. twice daily) were necessary, and even then suppression of lesions was less complete. In fact, the range of histological appearances varied from almost complete suppression, as described above, to a condition practically indistinguishable from that seen in the untreated animal.

It was found that aureomycin suspensions boiled for 5 minutes or allowed to stand for several days at room temperature were still effective; from the relevant literature it did not seem that aureomycin should be as stable as this. The question therefore arose as to whether aureomycin itself, or some impurity or breakdown product, was responsible for the therapeutic effect. Careful comparison was therefore made of the activity of aureomycin against grey lung virus and against a staphylococcal culture. (We express our thanks to our colleague Dr. A. T. Fuller for collaborating in these tests.) Aureomycin boiled for 5 minutes retained nearly all its activity against both test objects: but after boiling for one hour its activity against both was reduced to about 10 per cent. of its former value. It was also found that a sample of aureomycin "purified for intravenous use" was as effective as other samples. Histological study confirmed throughout the impressions gained by naked-eye inspection. We found, therefore, no reason to conclude that the activity was due to anything other than aureomycin itself.

Effect of delayed treatment.

Most chemotherapeutic agents are progressively less effective the longer the interval between infection and their administration. We were therefore surprised to find aureomycin so effective against grey lung virus when the beginning of treatment was delayed for five days. Subsequent tests showed that treatment might be postponed for three months and lesions could still be cleared up. Only small numbers of mice were available for these tests, but the results were consistent.

In these experiments 1 mg. doses were given daily over a period of 10 days, and mice were killed at the end of the course of treatment. The ± lesions represent small indefinite whitish areas in the lungs.

When the disease has been in existence for 2 to 3 months complete restoration of normal structure would scarcely be possible, for the usual alveolar pattern of the lungs by that time may well be distorted by the disintegration of alveolar cells and related interalveolar septa (Niven, 1950), and the disappearance of the large encircling cuffs of mononuclears is always followed by perivascular and

TABLE II.—*Effect of Aureomycin on Long-standing Infection* (6 *Experiments*).

Period after infection.																				
3½ months.	3½ months.				3 months.			3 months.				2 months.			2 months.					
Lesions in untreated mice.																				
+++	+++	+++	+++		+++	+++	+++	+++	+++			+++	+++	++	+++	++	0			
Lesions in treated mice.																				
0		±	±		0	0	0	±	±	±		0	±	±	0	0	0			

peribronchial fibrosis. In addition to these signs of pre-existing infection, all the animals of this series showed some degree of perivascular and peribronchial infiltration, which was, however, very slight in those in which the lungs were macroscopically normal. In the others an assortment of residual lesions was found amongst which areas of resolving consolidation, unaccompanied by invasion of polymorphs, were the most conspicuous. The most interesting feature was the complete disappearance of fluid exudate, except in those lungs in which considerable alveolar consolidation still remained, and even in those cases it was much reduced in amount. The epithelial hyperplasia also had undergone almost complete regression, and excessive mucus secretion had ceased. The surprising thing is, not that clearing-up of lesions was incomplete, but that so much resolution was produced so quickly in lesions of 3 months' standing.

In our earlier studies no evidence was found of development of immunity to grey lung virus in mice. We were now able to test whether mice cured by aureomycin were susceptible to re-infection with the virus ; this proved to be the case. Six mice were infected with grey lung virus, and cured by a course of six 1-mg. doses of aureomycin given between the fifth and eighth days after infection. Three were challenged with a dose of grey lung virus on the forty-first day, and all developed widespread lesions. These apparently developed as quickly and extensively as in animals following primary inoculation, and the appearances were in striking contrast to those of the series which were not re-infected ; two of these had trivial, doubtfully specific lesions, and the third none at all. Histologically, scanty foci of infiltration were noted in two, while in the third, the focus of infiltration found was trivial in comparison with the florid lesions of the reinfected group.

Two other experiments gave similar results.

Effect of aureomycin in eliminating virus.

Chemotherapy of infections of the psittacosis group with sulphonamides and other drugs may save the lives of mice or prevent lesions, but virus is not eliminated, nor, in the case of parakeets, are carriers cured (Quan, Meyer and Eddie, 1950). With aureomycin treatment of grey lung virus infection, however, virus seems to be extirpated. When mice are cured of infection, they may be killed at any time up to three months later, and the lungs will still be found to be clear : since active immunity seems not to be induced, this in itself indicates that there is no persisting pathogenic virus. We tried to recover virus from lungs of such cured mice, killed after eight and fifteen days : none was obtained, even after two serial passages. Even from a chronically-infected mouse, killed after a course of therapy carried out after infection, no virus was recovered.

Effect of Other Drugs.

Against most bacterial infections, chloromycetin (chloramphenicol) and aureomycin show a similar range of activity. Against grey lung virus, however, chloromycetin seemed quite impotent. Doses up to 5 mg. were given intraperitoneally twice daily from the first to ninth day after infection ; mice so treated showed lesions similar to those of controls.

Penicillin procaine in three divided doses, each of 33, 333 units, and streptomycin twice daily in doses of 0·2 to 0·4 mg., were equally ineffective. So, too,

was sulphamerazine (7·5 mg. daily intraperitoneally); indeed in some experiments we gave sulphamerazine routinely to all experimental animals, to keep under control the mouse pneumonitis (Nigg, 1942) virus which was prevalent in our stock mice. The results were again checked by histological study.

On the other hand, terramycin, in doses of 1 to 2 mg. daily for ten days, completely suppressed grey lung virus lesions; when given subcutaneously the local reactions produced were much less than those which aureomycin caused. Histologically the lungs of treated mice in this series showed a normal structure; a slight excess of polymorphs in the interalveolar septa and sinus catarrh and active phagocytosis of polymorphs in the tracheo-bronchial glands were the only abnormal findings.

It may be noted that Smadel and Jackson (1948) found chloromycetin effective against lymphogranuloma injected intraperitoneally in mice, but not when the virus was given intracerebrally. Aureomycin, however, was found by Wong and Cox (1948) to be active against intracerebrally inoculated lymphogranuloma in mice. We have found that in the case of intranasally inoculated mouse pneumonitis virus—another member of the psittacosis group—chloromycetin (2 mg. daily for one week) was ineffective, while aureomycin (1 mg. daily) inhibited lesions completely.

DISCUSSION.

There is very little evidence for the chemotherapeutic activity of any substance in experimental infections of animals with viruses outside the psittacosis-lymphogranuloma group. Eaton (1950) reports activity of aureomycin, but not chloromycetin, against his "atypical pneumonia virus" in cotton rats—a result which accords with favourable clinical reports concerning aureomycin on "primary atypical pneumonia" in man (Meiklejohn and Shragg, 1949). Activity of a number of substances against viruses in developing eggs is unfortunately not in general accompanied by demonstrable activity in infected mice.

The activity of aureomycin—and the inactivity of chloromycetin—against grey lung virus are therefore of special interest. It does not appear that this virus belongs to the psittacosis-lymphogranuloma group: it behaves differently immunologically; it is unaffected by penicillin or sulphonamides; elementary bodies are not demonstrable. The activity of aureomycin has led us to re-examine the question of whether "grey lung virus" is in truth a virus. So far we have found no evidence to the contrary, but are endeavouring to obtain further information, particularly by microscopical methods.

In a subsequent paper we describe a virus from cotton rats; this is possibly related to grey lung virus, and resembles it in its powers of long persistence and in its sensitivity to aureomycin (Andrewes and Niven, 1950).

We wish to thank Messrs. Lederle & Co., and Pfizer & Co., who made supplies of aureomycin and terramycin respectively available to the Medical Research Council. We also thank Mr. D. Busby, who carried out many routine inoculations of mice.

SUMMARY.

Infection of mice with grey lung virus is remarkably susceptible to therapy with aureomycin. Lung lesions, even chronic ones, are quickly resolved and

virus wholly eliminated. Cured mice are susceptible to re-infection with the virus. Terramycin is also active, but chloromycetin, penicillin, streptomycin and sulphamerazine are therapeutically ineffective.

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A VIRUS FROM COTTON-RATS: ITS RELATION TO GREY LUNG VIRUS

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DURING 1949 experiments were in progress, in collaboration with A. W. Gledhill, on transmission to rodents of a virus from infectious catarrh of cats. Greyish consolidation was observed in the lungs of inoculated cotton-rats (*Sigmodon hispidus*), and an agent was obtained which was transmissible to mice. Subsequently, similar lesions were found in the lungs of uninoculated adult cotton-rats and the same agent was obtained from them.

No conclusions could be drawn from transmission experiments to adult cotton-rats, as similar lesions could, subsequent to July, 1949, be found in the lungs of most of them. Recently-weaned cotton-rats were normally free from lesions, but transmission to mice was not difficult, and conclusions could be drawn more safely from experiments with that species.

EXPERIMENTAL METHODS.

Animals were inoculated intranasally under light ether anaesthesia, 0·05 ml. being given to mice and 0·2 ml. to cotton-rats. Mice were best killed for examination or passage 21 days after inoculation. Suspensions of their lungs were used for passage to more mice. Tissues for histological study were fixed in saturated mercuric chloride containing 4 per cent glacial acetic acid, air being removed promptly from lung tissue by evacuation under reduced pressure.

RESULTS.

The Disease in Cotton-rats.

A few adult cotton-rats killed and examined before July 1, 1949, had no macroscopic lung lesions, but of 23 killed in the following 2 months, all but 2 had greyish consolidation. The lesions were sometimes circumscribed, often at the hilum only, but more often they were diffuse and involved a large part of all lobes. Such cotton-rats usually appeared healthy at the time they were killed.

Of 12 recently-weaned cotton-rats killed during the same period, 2 had trivial lesions and only one extensive changes. At this time some of our normal cotton-rats were dying and extensive pneumonia was found in their lungs; it seems likely that the disease described was the cause of their deaths. The cotton-rat colony had been maintained at the National Institute for Medical Research (Mill Hill and Hampstead) since 1943; the condition was never noted between

1944 and 1946, when cotton-rat lungs were extensively used in research on scrub typhus (Buckland, Dudgeon, Edward, Henderson Begg, MacCallum, Niven, Rowlands, Van den Ende, Bargmann, Curtis and Shepherd, 1945); it seems therefore to have become endemic in a recognizable form since that time.

Histological picture in cotton-rats.

The conspicuous lung lesions were composed of two main elements, groups of consolidated alveoli and wide zones of cells of large lymphocyte type encircling blood vessels and bronchi of all orders. The consolidated areas were of varying size, some extending from the pleural surface to the hilum of the lung, smaller ones irregularly distributed between those limits and separated by greatly distended alveolar ducts. In less affected parts of the lung, where the normal micro-anatomical pattern was unaltered, the alveoli were dilated, and the alveolar septal cells enlarged and slightly increased in number. The alveolar consolidation, which was not accompanied by fibrosis, was due to the accumulation within the spaces of large cells with prominent vesicular nuclei rather poor in chromatin, and with abundant eosinophilic finely granular cytoplasm having a sharply defined border: occasional binucleate forms were found. These cells were derived from the interalveolar septa, and all stages from enlargement *in situ*, development of eosinophilic cytoplasm and separation from place of attachment on the alveolar wall could be readily made out. Phagocytosed material, generally the remains of polymorphonuclear leucocytes, was often present, and mitotic figures both in attached and separated cells were also seen. The perivascular infiltration was more extensive than the peribronchial, and occurred also around arterioles in relatively normal-appearing lung. Although it was so dense, the lymphatic channels were not obliterated by the infiltrating cells, and appeared as conspicuous channels with intact endothelial lining. The cells themselves belonged to the lymphocyte class, and had the characteristic deeply-staining nucleus and basophilic cytoplasm. Bronchial and bronchiolar hyperplasia, a striking feature of the disease in mice and in grey lung disease (Andrewes and Glover, 1945), was not conspicuous, nor was there any proliferation of the mesothelial lining of the pleura. The most outstanding difference, moreover, between this condition in the cotton-rat and grey lung disease was the complete absence of fluid exudate in alveoli and bronchi.

The Disease in Mice.

Mice inoculated intranasally with suspensions of cotton-rat lungs were killed after 8 days and showed no lesions. Serial passages at 8-day intervals did not lead to production of any evident disease. Those killed at 16 days or later showed small areas of whitish-grey consolidation, sometimes confluent, differing to the naked eye from those caused by mouse-pneumonitis, grey lung virus, influenza or other agents with which we were familiar. Passage at 3-week intervals proved to be convenient for propagating the infection in series: 8 serial passages have now been carried out. In later passages lesions have been somewhat more extensive, but deaths attributable to the infection itself have not occurred and the disease has remained of slow evolution; successful serial passage at shorter intervals has not proved possible. As with infection with grey lung virus, lung lesions have shown no tendency to spontaneous regression.

Histological picture in mice.

Microscopic changes can be seen as early as the third day. In the alveolar walls immediately beneath the pleura small accumulations of polymorphs are found, and the septal cells are enlarged and project into the lumen. By the seventh day this process, accompanied by marked capillary congestion, has extended throughout much of the lung, and small groups of consolidated alveoli occur beneath the pleura and also more deeply in the substance of the organ. The majority of the cells filling up the spaces are polymorphs, but detached septal cells with vesicular nuclei and eosinophilic cytoplasm are also present amongst them. In the bronchial and bronchiolar epithelium mitoses are quite numerous, and the pleural mesothelium also shows proliferative activity. Although fluid exudate into the alveoli is absent considerable perivascular oedema is present, and occasional lymphocytes and polymorphs have made their way into the scanty interstitial tissue surrounding the blood vessels.

By the tenth day the consolidated areas have enlarged, and the majority of the cells are still polymorphs; many, however, are degenerate, and phagocytosis by alveolar septal cells is taking place. Perivascular oedema of small and large arterioles, venules and main arteries and veins is generalized, and lymphocytes are beginning to accumulate around the vessels. Hyperplasia of the epithelium of the air-passages and pleura is more marked, and mitoses are more numerous. At the twentieth day the cellular picture in the area of consolidation has altered very considerably. Alveolar septal cells now predominate, and many contain phagocytosed remains of polymorphs in their cytoplasm. The degree of perivascular infiltration has increased tremendously, and the bronchi and bronchioles are also surrounded by cuffs of cells. Epithelial hyperplasia is also very conspicuous, and the cells are packed closely together and have a taller columnar form: no evidence of metaplastic process, however, has been found as a sequel to this proliferative activity. The consolidated areas vary very considerably in size: some involve only small groups of alveoli, others a considerable extent of lung substance. In the larger foci involving many alveoli unaffected alveolar ducts are always present. In the less-involved portions considerable numbers of air-containing alveoli, all emphysematous, surround small groups of consolidated alveoli. The absence of fluid exudate into the alveoli is at this stage the most striking point of difference between this condition and grey lung disease.

After five to six weeks, changes begin to appear in the alveolar cells in those areas of the lung where lesions most commonly appear first. The cytoplasm loses its eosinophilic staining and becomes very vacuolated; sometimes the vacuoles contain lipoid material, but more generally no specific substance can be identified. Shrinkage and distortion of the nucleus accompany the cytoplasmic alterations, and finally the whole cell disintegrates. This is followed by a secondary invasion of polymorphs. Epithelial hyperplasia never goes beyond a regular increased cellularity of the bronchial and bronchiolar lining, and ciliated cells maintain this feature, but increasing lymphocytic infiltration causes narrowing of the lumen and the membrane is thrown into folds, especially in the larger bronchi. Moreover, small groups of lymphocytes penetrate the basement membrane and accumulate between the cells without, however, causing ulceration of the surface. A similar phenomenon is also noted in the larger arteries where lymphocytes pass into the muscle coat.

The lungs of animals killed 6 to 10½ months after inoculation have a distinctly brownish mottled appearance, which is due to the presence of iron-containing material in many of the alveolar cells. Such iron-containing cells occur also in the lymphatic channels and in the tracheo-bronchial lymphatic glands. Thrombosed capillaries have been found in the larger consolidated areas, and occasional recent haemorrhages are seen. It is therefore likely that, although there is no fibrosis in the affected lungs, the pathological changes do eventually interfere with normal circulation, causing thrombosis and haemorrhage, the extravasated erythrocytes being ingested by the alveolar phagocytes.

Properties of the Causative Agent.

No cultivable agent has been obtained with the aid of a variety of bacteriological media, including those used for pleuro-pneumonia-like organisms. No elementary bodies have been found in stained smears (Gram and Machiavello stains), nor in histological preparations. Suspensions of mouse lungs have reproduced the disease in mice after dilution 1 in 100; higher dilutions were ineffective. Intraperitoneal and subcutaneous injection did not lead to infection. No agent was recovered from a pool of spleen, kidney and liver of infected mice having extensive lung lesions. Filtration, after clarification through paper pulp, has been successful in one of two attempts through gradocol membranes of average pore diameter 0·7μ; 0·5μ membranes held the agent back. Virus has been recovered from the lungs of mice infected 3, 5½ and 10½ months previously. Spontaneous transfer of infection from mouse to mouse within a cage could not be demonstrated.

Effect of Chemotherapy.

Sulphamerazine (7·5 mg. daily intraperitoneally) and chloromycetin (2 mg. daily intraperitoneally) were apparently without effect on the course of the infection. On the other hand, aureomycin and terramycin were highly effective, as they were against grey lung virus (Andrewes and Niven, 1950). In one experiment the potency of these two drugs was compared, 7 daily doses being given subcutaneously, beginning on the day of inoculation: mice were killed after 21 days.

TABLE I.—*Effect of Drugs on Cotton-rat Virus.*

	Aureomycin.	Terramycin.	Controls.
2 mg. dose	..	0 0 0 0	(no drug)
1 mg.	0 0 0 0	0 0 0 0	++ ++ ++
0·5 mg.	0 0 0 0	± 0 0 0	++ ++
0·25 mg.	0 0 0 0	++ + +	

++, +, ±, 0 indicate extent of lung lesions.

Histologically, lungs of mice treated with aureomycin and terramycin showed an almost normal structure. In the former group a scanty infiltration of lymphocytes was found in a few areas, and a somewhat greater degree is present in the terramycin series. In the latter, in addition, polymorphs in slight excess were detected in the alveolar walls. The significance of these appearances is discussed below.

Aureomycin thus seems more potent than terramycin. The conditions under which it was effective were not investigated in such detail as in tests with grey lung virus (Andrewes and Niven, 1950). It seems, however, that as with that virus, mice cured with aureomycin are susceptible to re-infection, little or no active immunity having been evoked (Table II).

TABLE II.—*Re-infection with cotton-rat Virus of Mice Cured wtih Aureomycin.*

Untreated mice.	Aureomycin-treated mice (no challenge).	Aureomycin-treated mice challenged with fresh virus.	Normal mice receiving same virus as for challenge inoculum.
* ++ ++	0 0 0	++ ++ ++ ++	++ ++ ++
++ ++ ++	± ± ±	++ ++ ++ ++	++ ++ ++

++ = extensive grey lesions ; ± = a few tiny grey spots ; 0 = no lesions ; * = non-specific death.

The treated mice received aureomycin from the twenty-ninth to thirty-sixth day after infection ; challenge was on the fifty-third day. Mice were killed and examined on the eighty-second day.

In the aureomycin-treated mice, in this experiment, only one animal was free from alveolar lesions. Of the remaining five, three showed small areas of alveolar consolidation and lymphocytic infiltration just visible macroscopically, while in two, lesions were only detected on histological examination. Although it is impossible to assess accurately the age of these foci, the cytological characters of these cells did suggest that they had been developing slowly over a period of some weeks. In all the animals, however, they were insignificant in size compared with the lesions found in the untreated control group, in the group challenged after treatment, and in the normal mice which were inoculated simultaneously with the challenged group. These findings make it impossible to assume that the polymorph and lymphocytic infiltration found microscopically in the first aureomycin and terramycin treated series represent the terminal stages of the disappearance of the pathological changes ; it seems more probable that they have to be interpreted as the tissue reaction to a very small amount of virus which has resisted treatment.

DISCUSSION.

The foregoing account makes it clear that in two important respects this cotton-rat virus resembles grey lung virus (Andrewes and Glover, 1945). The virus and the lesions it produces tend to persist in lungs of infected mice with very little change over periods of months. Further, both viruses are very sensitive to the action of aureomycin, but not chloromycetin ; both infect mice and cotton-rats, and finally, both have turned up in the same laboratory.

Immunological comparison was not possible, since the grey lung virus has not been shown to provoke active immunity nor antibody formation, and the cotton-rat virus, so far as it has been studied, behaves likewise. Other differences are perhaps only of degree. The disease due to cotton-rat virus is of slower evolution ; the lesions differ macroscopically and microscopically—in particular the oedema of grey lung infection is not noticeable ; the virus goes to a low titre

(10^{-2} versus 10^{-5} or 10^{-6}), and there are other differences which may be associated with its lower level of activity. One cannot deny the possibility that the viruses represent different phases of pathogenicity of a single virus. On the other hand, they may be two representatives of a family of viruses having in common the properties of sensitivity to aureomycin (but not to chloromycetin, penicillin or sulphonamides), and of causing disease of remarkable chronicity in the host. A virus ("Potash") isolated by K. Goodner (1950, personal communication) seems to be similar, and may be another biological variant or another member of the family. Dr. H. Rose (1950, personal communication), of New York, informs us that he also has isolated a similar agent from cotton-rats. It is likely also that the virus is identical with one of the cotton-rat viruses which Eaton, Meiklejohn and van Herick (1944) have mentioned. Should this virus prove to be intimately related to grey lung virus, a clue would be afforded to the origin of that virus—a thing hitherto obscure. Possibly the virus now described is an endemic virus of cotton-rats, and the grey lung virus a derivative of it which had acquired new properties by spontaneous transfer to, and passage through, mice at the time when Andrewes and Glover isolated it in 1944.

SUMMARY.

A virus transmissible to mice has been recovered from lung lesions in normal cotton-rats; some of its properties are described. It differs from grey lung virus in its lower level of activity in mice, in the slower evolution of the lesions produced and in their naked-eye and histological appearances. It resembles grey lung virus in the remarkable chronicity of the disease produced, and in its susceptibility to aureomycin and terramycin. The two agents may be biological variants of a single virus, or two members of an unusual group of organisms.

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THE RELATION BETWEEN ANTITOXIC RESPONSES TO PROPHYLACTIC INJECTIONS OF DIFFERENT ANTIGENS.

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In any group of animals injected with the same dose of a given prophylactic, the resulting antitoxic values differ within the group according to the capacity of the individual animals to respond to the particular kind of stimulation given. When two relatively large doses, for example 1 Lf of diphtheria A.P.T., are injected into normal guinea-pigs, a single population of values is obtained ; these values are normally distributed and show a small scatter, because the dosage is sufficient to produce a good titre of antibody even in the poor responders. If the dose is considerably reduced, however, the resulting antitoxic values may fall into two populations, and the poor responders become clearly distinguishable from the good responders.

The reasons for differences in response to immunization are not known : poor response may at times be caused by a temporary condition of the animal such as adventitious infection, or there may exist some inherent physiological incapacity to respond well to a given type of stimulation.

During the period 1938 to 1945 all incoming normal horses bought by these laboratories were given two injections each of diphtheria A.P.T., tetanus A.P.T., *Clostridium oedematiens* A.P.T. and *Clostridium septicum* toxin. This procedure was adopted as a routine measure at a time when it was anticipated that many hundred horses would be required for the production of tetanus and gas gangrene antitoxins. Basal immunity would thus be established in all horses, and the good responders to the various antigens could be allocated for suitable hyper-immunization courses after a period of rest. Antitoxin titrations were therefore made on blood samples taken 14 days after the second injection, which was given 4 weeks after the first. Tests for diphtheria and *Cl. septicum* antitoxin were made by the guinea-pig intracutaneous methods, and for *Cl. tetani* and *Cl. oedematiens* antitoxins by the mouse subcutaneous method. All tests were done at approximately 100 per cent differences except those for *Cl. oedematiens* antitoxin, which were done at approximately 4-fold differences, the levels titrated being 5, 20 and 100 units per ml. This wide testing was decided upon to avoid undue expenditure of labour and animals : all these antitoxins can easily be titrated at 10 per cent differences when required. This paper is concerned with an analysis of the responses of horses to these injections over part of this period, and we have found some correlation between the responses of individual animals to the various antigens.

Table I compares the responses of 650 horses to two injections each of 250 Lf doses of diphtheria A.P.T. and 100 Lf doses of tetanus A.P.T. In addition to the blood samples taken 14 days after the second injection of antigens, the values

of which are shown in Table I, an earlier sample was taken 10 days after the first injection for the purpose of testing potential immunity. It was found (Barr, 1950) that approximately 40 per cent of 1000 horses with no circulating antitoxin possessed some potential immunity to diphtheria. We also tested the early blood samples for tetanus antitoxin because of the possibility that certain horses had received prophylactic injections of tetanus toxoid or A.P.T. before arrival at these Laboratories. From the results of these tests we formed the opinion that very low-grade potential immunity to tetanus might exist in certain normal horses as a result of natural stimulation, because feeble responses were encountered in some of the animals tested. These were thought to be much too low to be accounted for by the existence of potential immunity following injections of prophylactic, though it is possible that these horses were very poor responders which had only received one injection previously. Considerable responses occurred in some horses to the first injection of diphtheria A.P.T., and these results have already been analysed and reported by one of us (Barr, 1950). Those horses that showed potential immunity to diphtheria have been excluded from Table I.

TABLE I.—*A Comparison of the Responses of Normal Horses to Two Injections each of Diphtheria A.P.T. and Tetanus A.P.T. The Horses had no Normal Immunity to Diphtheria.*

Tetanus response (U.S.A. units/ml.).	Diphtheria response % with titres over— 0·01	0·1	1·0 unit/ml.	Diphtheria response (median).	Number of horses.
Under 1	55·3	36·8	7·9	0·0252	38
1	80·8	65·4	19·2	0·260	26
2	94·9	81·0	27·8	0·510	79
5	98·0	92·9	48·5	0·954	99
10	98·4	97·8	59·8	1·27	184
20	98·0	93·4	63·6	1·48	151
50	98·0	96·0	66·0	1·66	50
Over 50	100·0	100·0	87·0	3·04	23
					Total 650

It will be seen that there is a definite correspondence between the antitoxic responses of horses to the two antigens, for 44·7 per cent of horses failing to produce 1 unit of tetanus antitoxin produced less than 0·01 unit of diphtheria antitoxin, and only 7·9 per cent produced over 1 unit per ml. At the other end of the table, a very high proportion of horses producing 50 units per ml. or more tetanus antitoxin also responded well to the diphtheria antigen. The median antitoxin value of the diphtheria titres for each group showed a steady increase in parallel with the tetanus response. Median values are recorded here because some of the diphtheria antitoxic values were below the lowest level detectable in the test (0·0005 unit per ml.), thus precluding the use of geometric means.

A similar correspondence between the responses of 797 horses to tetanus A.P.T., *Cl. oedematiens* A.P.T. and *Cl. septicum* toxin is shown in Table II. In this comparison the same batch of each antigen was used throughout. The results have been grouped according to the tetanus response, and the percentages in each group that exceeded 5 units of *Cl. septicum* and 20 units of *Cl. oedematiens* are

TABLE II.—*A Comparison of the Responses of Normal Horses to Two Injections each of Tetanus A.P.T.*, Cl. septicum *Toxin and* Cl. oedematiens *A.P.T.*

Tetanus response (U.S.A. units/ml.).	Percentage of horses with antitoxic values above— 5 units *septicum*.	Total number.	20 units *oedematiens*.	Total number.
Under 5	5	109	30	111
5, 10	21	400	76	400
20 and over	42	288	91	286
		797		797

given. With increasing antitoxic response to tetanus A.P.T. the percentage exceeding the chosen levels for the other two antitoxins also increased.

The responses to these three antigens have been further analysed in relation to the normal state of immunity to diphtheria for over 2600 horses. The same batches of each antigen were not used for all these horses. This additional analysis was made because it appeared probable that if the majority of aged horses have been in contact with diphtheria at some time, the apparently non-immune group (some of which however might be potentially immune) would include a higher proportion of poor responders than the immune group. These results are shown in Table III ; we define horses without potential immunity as

TABLE III.—*The Responses of Normal Horses to two Injections each of Tetanus A.P.T.*, Cl. septicum *toxin and* Cl. oedematiens *A.P.T. in Relation to their State of Normal Immunity to Diphtheria.*

Diphtheria immunity.	Tetanus response. Per cent <1 unit	Tetanus response. Per cent >20 units.	Total no.	Septicum response. Per cent <1 unit	Septicum response. Per cent >5 units.	Total no.	Oedematiens response. Per cent <5 units.	Oedematiens response. Per cent >20 units.	Total no.
Non-immune	5·8	41·3	757	33·0	29·4	674	25·4	43·8	685
Potentially immune	3·1	45·0	515	24·3	34·5	464	23·5	46·0	476
Immune	3·2	47·6	1507	25·5	40·0	1489	17·3	57·0	1586
Total			2779			2627			2747

those with no detectable antitoxin on arrival that failed to respond in 10 days to the first injection of A.P.T. The potentially immunes also had no antitoxin on arrival but showed a response 10 days after their first injection, while the immune horses all had circulating antitoxin on arrival. The figures in Table III show that for each of the three antitoxins tested there were fewer low values and more high values in the group with natural immunity than in the non-immune group, but the differences are not all very great. The presence or absence of circulating antitoxin or potential immunity to diphtheria in normal horses must depend upon the amount of earlier natural stimulation and the lapse of time since this occurred, as well as upon the capacity of the animal to respond (Barr and Glenny, 1945 ; Barr, 1950). The results in Table III suggest, however, that poor response plays an appreciable part in the failure to develop immunity as a result of natural infection.

We have occasionally encountered extremely resistant animals, and quote here the example of a horse which, after five injections of *Cl. tetani*, *Cl. oedematiens* and *Cl. septicum* antigens given at monthly intervals, failed to produce 1 unit per ml. of any of the corresponding antitoxins. After six injections given over a period of 7 months, this horse produced a tetanus antitoxic titre between 2 and 5 units per ml.; the *Cl. septicum* value was still below 1 unit and the *Cl.* oedematiens value under 5 units per ml. (this was not tested lower). Eight injections were given before 1 unit per ml. of each antitoxin was developed : the final titres were tetanus 20 units, *Cl. septicum* 1 to 5 units and *Cl. oedematiens* 5 to 20 units per ml. Two other horses, both of which required six injections before 1 unit per ml. of these antitoxins was produced, received courses of hyperimmunization with *Cl. welchii* (*perfringens*) toxin. In neither case was antitoxin of useful titre produced. The first horse referred to in this paragraph was used for the production of antibacterial serum. During the early part of the period under review we became convinced that horses responding badly to these prophylactic injections were, as a general rule, poor responders to hyperimmunization with any kind of toxin or toxoid. In consequence of this, few such horses were used for the production of antitoxic sera.

DISCUSSION.

The production of antitoxin by an animal in response to two injections of antigen depends upon several factors. These can be described generally as the quantity and quality of injected antigen and the immunity state and power of response of the animal. In any group of animals given the same dose of the same batch of antigen there may be a considerable scatter in the resulting antitoxic titres, even in the complete absence of basal immunity in any animal at the start of the experiment. This is the case when guinea-pigs are injected with diphtheria prophylactic : the short and secluded life of guinea-pigs bred for experimental purposes precludes the possibility of establishing natural basal immunity to such infections as diphtheria, tetanus, *Cl. oedematiens* and *Cl. septicum*. Passive immunity, however, is transmitted to young born of actively immunized does, via the placenta ; consequently only normal guinea-pigs can be used for breeding purposes in laboratory work.

The scatter of values among guinea-pigs after two injections of prophylactic may be considerable if the dosage is small or the quality poor, but is very limited if large doses of good quality antigen are given. Work on horses, however, is complicated by the existence of naturally acquired immunity to certain types of infection. In the past, horses had ample opportunity of acquiring considerable immunity to diphtheria, probably through infection of scratches. Many horses also show natural immunity to *Cl. welchii* (*perfringens*).

It is known that cows and sheep in certain districts acquire immunity, shown as circulating antitoxin, to *Cl. tetani*, *Cl. oedematiens* and *Cl. septicum*. It appears probable, or at least possible, that horses may acquire a similar natural immunity to a lesser degree. At one time we were of the opinion that horses did not acquire natural immunity to tetanus, but more extensive experience in the recent war, involving over 5000 horses, has led us to the conclusion that the number we have found with feeble potential immunity could not be explained by previous inoculation with tetanus toxoid. In the work recorded in this paper there is therefore an

uncontrolled variable in the former immunological history of the horses : variables due to antigenic differences have been avoided in Tables I and II by using only those results that were obtained with the same batches of antigen.

Our results show that there is some general correlation between the responses of individual horses to injections of two or more antigens. The arguments given above suggest that a very close correlation could not be expected, but the results indicate that some individuals in a species may possess a poor capacity to produce all kinds of antibodies associated with a particular serum protein. As yet we have no evidence to show whether the same general principle applies to man, but we have found instances in which individuals responded well to tetanus immunization and badly to diphtheria immunization. We are of the opinion that this can be explained by the use of adequate dosage in tetanus prophylaxis and insufficient dosage in diphtheria prophylaxis in adults. It appears possible that there is a general tendency in an individual to respond well or badly, but that a refractory state may be either temporary or permanent. It may be due to some physiological deficiency, or it may be the result of " crowding out " by responses to some natural stimulation. Poor responses to one antigen in an individual easy to immunize against other antigens may at times be due to the earlier establishment of basal immunity by a slightly denatured antigen.

In our experience of immunization of young babies against diphtheria, in collaboration with Dr. Randall (1950), we rarely found poor responders, so long as the passive antitoxin derived from the mother was insufficiently high to interfere with the action of the prophylactic. The generally good response can be attributed to use of an adequate system of dosage. This may not be the sole reason for the success of immunization in very early life, because young babies have had fewer opportunities of acquiring infections which would limit their capacity to respond to prophylactic injections.

SUMMARY.

Many hundred horses received two injections of diphtheria, tetanus, *oedematiens* and *septicum* prophylactics. Antitoxin titrations were made upon samples of serum withdrawn 14 days after the second injection, given 4 weeks after the first. Evidence is given showing some correlation between the titres of the different antitoxins.

A few horses were found to be very refractory to immunization, and one horse is recorded which, after receiving 5 monthly injections of tetanus, *oedematiens* and *septicum* prophylactics, failed to produce 1 unit of any of the corresponding antitoxins. Of 650 horses given two doses, only 5·8 per cent failed to produce this amount of tetanus antitoxin.

Horses naturally immune to diphtheria appeared to respond slightly better to injections of the three other prophylactics than horses with no such natural immunity.

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SEROLOGICAL STUDY OF THE SOLUBLE ANTIGENS OF VARIOLA, VACCINIA, COWPOX AND ECTROMELIA VIRUSES.

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USING hyper-immune rabbit sera absorbed with elementary body suspensions Downie (1939) demonstrated differences in the antigenic structure of the viruses of vaccinia and spontaneous cowpox. He suggested that these differences might reside in the heat-labile antigenic components. In a later paper the immunological relationships of the viruses of variola, alastrim, vaccinia, cowpox and ectromelia were studied by complement fixation and inhibition of complement fixation methods (Downie and Macdonald, 1950). Immune animal serum prepared against any one virus was found to be capable of fixing complement in the presence of antigens derived from any other member of the group, but reactions were stronger with homologous than with heterologous antigens. Antisera prepared in fowls were considered to be more specific than animal antisera, and these were employed in the inhibition of complement fixation method. By this technique the viruses of variola, alastrim and vaccinia could not be clearly distinguished, but cowpox and ectromelia viruses were found to be more closely related to each other than either was to variola or vaccinia virus. Since the inhibition of complement fixation is a sensitive technique for *in vitro* serological studies on this group of viruses it was decided to re-investigate their relationships with particular reference to heat-stable antigens.

MATERIALS AND METHODS.

These have been described in detail in a previous paper (Downie and Macdonald, 1950).

Animal antigens, free from virus, were derived from human smallpox crusts, from dermal pulp of rabbits infected with rabbit-adapted strains of vaccinia and cowpox viruses and from mouse ectromelia liver.

Soluble egg antigens were prepared from infected chorio-allantoic membranes, using strains of virus which had been passed only in eggs.

The animal antisera were obtained from rabbits hyper-immunized by injections of elementary body suspensions of rabbit-adapted cowpox and vaccinia viruses.

The fowl antisera were from fowls immunized with living suspensions of egg-adapted virus obtained from infected chorio-allantoic membranes.

Complement fixation tests.

Complement fixation tests were carried out using two-fold dilutions of antigen and of antiserum and 3 M.H.D. of azide-preserved complement in unit volumes

of 0·2 ml. Tests were kept at 4° C. overnight before adding 0·4 ml. of sensitized cells, which were prepared by mixing equal volumes of amboceptor, containing 5–6 M.H.D., and 5·0 per cent washed sheep cells. The racks were then placed in a water-bath at 37° C. for 30 minutes, and allowed to stand 1–2 hours at room temperature before recording the results. The last tube showing 50 per cent haemolysis *or less* was taken as the end point.

Complement fixation inhibition tests.

In the complement-fixation inhibition tests 0·1 ml. of antigen, in suitable dilution, was first added to the tubes, followed by 0·1 ml. of complement containing 3 M.H.D. and 0·2 ml. of fowl antiserum dilutions. After shaking, these mixtures were allowed to stand at room temperature for 4 hours before adding 0·2 ml. of rabbit antiserum. The method of selecting appropriate concentrations of antigen and rabbit antisera for these tests is described in the previous paper. In general the amounts were twice the minimal fixing dose. The tests were then placed in the refrigerator overnight, and completed in the same way as in the ordinary complement fixation method. The last tube showing 50 per cent haemolysis *or more* was taken as the end point.

RESULTS.

Comparative complement-fixation tests with unheated and heated antigens.

The soluble antigens used in the complement fixation tests were divided into two portions, one being heated at 68–70° C. for 1 hour—a temperature sufficient to destroy the heat-labile component of the LS antigen of vaccinia virus (Craigie and Wishart, 1936). The heated and unheated antigens were then tested at the same time, all other materials and conditions being identical. While it was known that variola, vaccinia and cowpox viruses contained heat-labile components, no similar information was available for ectromelia and alastrim viruses. The results of comparative tests using animal antigens are shown in Fig. 1.

It can be seen that both of the immune rabbit sera fixed complement with all the antigens, although reactions were stronger with homologous than with heterologous antigens.

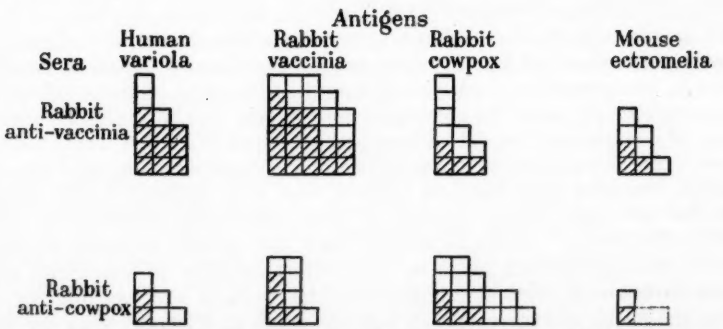

FIG. 1.—Complement fixation tests using unheated and heated animal antigens. The vertical divisions in the figures represent two-fold dilutions of antigen. The horizontal divisions in the figures represent two-fold dilutions of serum. In each figure the total area represents extent of positive reactions with unheated antigens; cross-hatched squares indicate positive reactions with heated antigens.

The cross-hatched areas in the figure show that heating reduced the capacity of each antigen to fix complement in the presence of anti-vaccinial and anti-cowpox rabbit sera. Heating the antigens did not affect the specificity of the reactions, although each serum fixed complement in the presence of all the heated antigens. It is apparent that mouse ectromelia antigen behaved in a similar manner to the others, showing that ectromelia also has a heat-labile and a heat-stable component. While the degree of reduction in complement fixing titres by heating varied from one antigen to another, this does not necessarily imply that the amount of the heat-labile component in the different viruses varies in a corresponding manner.

Tests were also carried out with unheated and heated antigens derived from infected chorio-allantoic membranes. The results of tests using a constant amount of antiserum are shown in Table I. Heating reduced the complement

TABLE I.—*Antigen Titres obtained by Complement Fixation with Unheated and Heated Egg Antigens and Rabbit Antisera.*

Immune rabbit serum.	Antigens.	Egg antigens. Variola.	Alastrim.	Vaccinia.	Cowpox.	Ectromelia.
Anti-vaccinial 1/80.	Unheated	1280	320	1280	320	40
	Heated	320	160	320	80	20
Anti-cowpox 1/40.	Unheated	640	320	320	160	40
	Heated	80	80	160	80	20

fixing titre 2–8-fold, and again it can be seen that the specificity of the egg antigens has not been affected by heat treatment. In this table it is also apparent that ectromelia egg antigen, when heated, loses a portion of its complement fixing capacity, and the same is true for alastrim egg antigen. This supports the view that ectromelia and alastrim antigens have heat-labile and heat-stable components and, in this respect, are similar to variola, vaccinia and cowpox.

Complement fixation inhibition tests using heated antigens.

The complement fixation inhibition test is an indirect method of determining the antibody content of fowl or other types of antiserum which do not fix complement in the presence of antigen in the ordinary type of test. The fowl sera combine specifically with the appropriate antigens, and in doing so prevent the fixation of complement on the subsequent addition of known fixing mammalian antisera. By this technique it was shown in the previous paper that the antigens of variola, alastrim and vaccinia viruses were very similar, while cowpox and ectromelia are more closely related to each other than either is to the other viruses in the group. Tables II and III show the results of inhibition tests using unheated and heated egg antigens and rabbit antisera as the fixing mixtures. In these tables each vertical column gives the result of one test, but, for each egg antigen the fixing mixtures varied, and the titres in different tests are not comparable. It can be seen that the fowl antisera show, by their capacity to inhibit fixation, a narrower range of activity than mammalian antisera. The fowl antisera prepared against variola, vaccinia and alastrim virus were found to be capable of inhibiting fixation with all the heated and unheated antigens, and there

TABLE II.—*Complement Fixation Inhibition Titres of Fowl Antisera using as Fixing System Rabbit Anti-vaccinia Serum and Egg Antigens.*

Immune fowl sera.	Antigens. Variola. U	Variola. H	Alastrim. U	Alastrim. H	Vaccinia. U	Vaccinia. H	Cowpox. U	Cowpox. H	Ectromelia. U	Ectromelia. H
Cowpox	—	—	—	—	—	—	80	40	80	80
Vaccinia	40	20	80	20	160	40	40	40	80	40
Ectromelia	—	—	—	—	—	—	40	20	320	40
Variola	320	320	640	80	160	80	160	40	640	160
Alastrim	320	320	320	320	320	320	320	20	320	80

U = unheated; H = heated; — = no inhibition by fowl serum dilution of 1/10.

TABLE III.—*Complement Fixation Inhibition Titres of Fowl Antisera using as Fixing System Rabbit Anti-cowpox Serum and Egg Antigens.*

Immune fowl sera.	Antigens. Variola. U	Variola. H	Alastrim. U	Alastrim. H	Vaccinia. U	Vaccinia. H	Cowpox. U	Cowpox. H	Ectromelia. U	Ectromelia. H
Cowpox	—	—	—	—	—	—	160	40	20	40
Vaccinia	40	80	80	20	20	80	160	80	20	40
Ectromelia	—	—	—	—	—	—	—	20	40	80
Variola	320	320	160	80	160	160	160	40	80	160
Alastrim	320	320	160	80	160	320	160	20	80	80

U. = unheated; H = heated; — = no inhibition by fowl serum diluted 1/10.

is therefore no evidence that these antisera could clearly differentiate the three viruses by this technique. Cowpox and ectromelia fowl antisera on the other hand were incapable of inhibiting fixation when the antigens were those of variola, vaccinia and alastrim. These two fowl antisera did, however, inhibit fixation with the homologous antigen or with the other antigen of the pair. In other words, these fowl antisera do not appear to possess all the antibodies necessary to bind completely the antigens of variola, vaccinia and alastrim. The tables show that the results are similar whether heated or unheated antigen was used, suggesting that the differences detected by this technique do not reside in the heat-labile component. Essentially similar results were obtained with unheated and heated antigens of mammalian origin.

DISCUSSION.

Smadel and Hoagland (1942) found that samples of LS antigen were serologically indistinguishable, whether they were derived from human crusts or chorio-allantoic membranes infected with variola virus or from the tissues of animals or eggs infected with vaccinia virus. In our experiments the reactivity of the soluble antigens used was almost certainly due to LS antigens. In complement fixation tests with unheated antigens from the viruses of variola, alastrim, vaccinia, cowpox and ectromelia, we were also unable to differentiate clearly the various viruses, although homologous antigens and antisera always gave stronger fixation (Downie and Macdonald, 1950). Because of a suggestion by Downie (1939) that the serological differences which he had found between

vaccinia and cowpox viruses might reside in the heat-labile components of the antigens, tests have been made with heated antigens. Heating to 70° C. for 1 hour reduced the fixing capacity of the antigens from all the viruses studied, and it must therefore be assumed that alastrim and ectromelia viruses, like the other viruses in the group, have heat-stable and heat-labile components. The heating of the soluble antigens did not appear to affect the serological specificity in the complement fixation reactions as compared with native antigens. The inhibition of complement fixation method using fowl antisera had previously been found to be a more sensitive test for the detection of antigenic differences in this group of viruses. When the method was applied, using heated soluble antigens, either of mammalian or egg origin, the results were almost identical with those obtained with native antigens, indicating that the heat-stable antigens retain such specificity as is possessed by the native antigens. The experiments again showed that the soluble antigens of cowpox and ectromelia viruses are more closely related to each other than they are to those of the variola-vaccinia group.

SUMMARY.

The antigenic relationships of the viruses of variola, alastrim, vaccinia, cowpox and ectromelia have been studied, using heated soluble antigens for serological tests. After being heated at 68–70° C. for 1 hour, the soluble antigens of ectromelia and alastrim viruses, like those of variola and vaccinia, still retain a portion of their potency. This indicates that they both have heat-labile and heat-stable antigenic components.

Complement fixation tests with rabbit antisera and heated soluble antigens of mammalian or egg origin did not serve to differentiate clearly the members of this group of viruses. Reactions were, however, stronger with homologous than with heterologous systems.

By the inhibition of complement fixation method with heated antigens it was confirmed that the soluble antigens of cowpox and ectromelia viruses bear a closer relationship to each other than either does to the variola-vaccinia viruses. The heated antigens retain the same serological specificity as do the native antigens, indicating that such antigenic differences as are demonstrable are not specifically associated with the labile component of the LS antigens.

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THE VIRUSES OF VARIOLA, VACCINIA, COWPOX AND ECTROMELIA.—NEUTRALIZATION TESTS ON THE CHORIO-ALLANTOIS WITH UNABSORBED AND ABSORBED IMMUNE SERA.

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In a previous paper we recorded the results of neutralization tests with the viruses of variola, vaccinia, cowpox and ectromelia and corresponding immune sera (McCarthy and Downie, 1948). Four strains of variola, two strains each of vaccinia and cowpox and one strain each of alastrim and ectromelia viruses were examined. Immune sera from immunized fowls and rabbits and from man were used in neutralization tests made by inoculating serum-virus mixtures on the chorio-allantois of chick embryos or into the skin of rabbits. In the tests on the chorio-allantois, undiluted immune sera prepared against each of the virus strains was tested against a constant amount of virus, and in the rabbit skin constant amounts of sera were tested against varying amounts of cowpox and vaccinia viruses. These cross-neutralization tests showed the close relationship of the strains studied and failed to demonstrate distinct differences among them. In an attempt to disclose such differences we have made an estimate of the neutralizing antibody in immune sera by testing five-fold dilutions of the sera against homologous and heterologous viruses. In this extension of the previous work only immune sera prepared in fowls have been studied and all neutralization tests have been made on the chorio-allantois of chick embryos. In addition, we have carried out absorption of immune fowl sera with virus suspensions and tested the absorbed sera for neutralizing power.

MATERIAL AND METHODS.

The strains of virus and the immune fowl sera were those described in the previous paper. With the exception of one vaccinia and one cowpox strain used for the absorption of sera, only strains propagated on the chorio-allantois were studied in the present experiments.

Absorption of sera.

The immune sera prepared in fowls against cowpox, vaccinia, ectromelia and one strain of variola were absorped with virus suspensions. The absorbing suspensions of cowpox and vaccinia viruses were prepared from dermal infections in the rabbit with strains which had been propagated only in this animal, as by this method relatively pure and concentrated suspensions more suitable for absorption of sera were readily obtained. The absorbing suspensions of ectromelia and variola were prepared from infected chorio-allantoic membranes.

A suspension of vaccinia elementary bodies representing the yield from two rabbits was divided into two parts. One was equally distributed in five centrifuge tubes which were spun in an angle centrifuge at high speed to deposit the virus. After the supernatant fluids had been discarded 1·5 ml. of immune fowl serum was added to four tubes, one serum to each tube, and normal fowl serum to the fifth. The virus deposit was resuspended in the serum and the tubes left overnight in the refrigerator (2–4° C.). Next day the virus was deposited by centrifugation, and the supernatant fluids removed and treated in the same way with the virus from the remaining part of the original suspension. After the second absorption the mixtures were spun at high speed for 1½ hours and the supernatant absorbed sera removed and heated at 57° C. for 30 minutes to inactivate any virus remaining in the serum. Similar volumes of sera were absorbed with cowpox elementary body suspension from infected rabbits in a similar manner. The treatment resulted in only slight dilution of the sera.

The ectromelia virus suspension used for absorption was prepared by differential centrifugation from 30 infected chorio-allantoic membranes. The same five sera were absorbed as before in two stages, but because of the smaller amount of virus available only 0·1 ml. of each serum (diluted to 1·0 ml.) was used, and the volume was finally made up to 1·5 ml. with saline, so that the final dilution of absorbed serum was 1 in 15. In the same way variola virus from 30 membranes was used to absorb 0·1 ml. of each of the five sera. Samples of each serum were similarly treated with egg-grown herpes simplex virus. Absorption with this virus did not reduce the titre of any of the sera for variola virus which was the only virus-tested.

Technique of neutralization tests with absorbed and unabsorbed sera.

The sera which had been absorbed with vaccinia and with cowpox viruses were tested together with the corresponding unabsorbed sera against one virus at a time. The test virus suspension had been prepared from infected chorio-allantoic membranes and titrated for virus content. The dilution of virus suspension used was such that the mixture with normal serum produced between 50 and 200 lesions per egg ; 0·3 ml. of virus dilution was added to 0·3 ml. of each serum, and after the mixtures had been left at room temperature for 30 to 40 minutes each was distributed on the chorio-allantois of five or six 12-day embryos. In a similar manner the unabsorbed sera diluted 1 in 15 and the sera absorbed with variola and ectromelia were tested together against one virus at a time. Inoculated eggs were opened after 3 days, the membranes excised and placed in formol saline for the counting of lesions. In recording the results the average number of lesions per egg for any one serum-virus mixture has been expressed as a percentage of the average number of lesions produced by the same virus mixed with unabsorbed or absorbed normal fowl serum.

RESULTS.

I. *Neutralization of Viruses by Dilutions of Homologous and Heterologous Immune Fowl Sera.*

In each experiment the four sera, in five-fold dilutions, were tested simultaneously against one virus. To the dilutions of sera the virus suspension was added at intervals so that thirty minutes should elapse before the inoculation of each

mixture into eggs. Five or six 12-day embryos were inoculated with each mixture. The results of the tests with four viruses are shown in Tables I to IV.

Table I shows that all four sera neutralized cowpox virus to fairly high dilution, the variola antiserum being the strongest. Vaccinia virus (Table II) was in-

TABLE I.—*Neutralization of Cowpox Virus by Immune Fowl Sera.*

Average number of lesions on chorio-allantois expressed as percentage of controls.

Serum dilutions.	Antisera.			
	Cowpox.	Vaccinia.	Ectromelia.	Variola.
1/10	6	3	1	2
1/50	4	5	5	1
1/250	17	5	7	4
1/1250	50	47	40	19
1/6250	58	76	64	56

Figures in Tables I to IV and VI and VII represent average number of lesions on 5 to 6 eggs expressed as a percentage of average numbers of lesions on 5 to 6 eggs inoculated with the same amount of virus and normal fowl serum.

TABLE II.—*Neutralization of Vaccinia Virus by Immune Fowl Sera.*

Average number of lesions on chorio-allantois expressed as percentage of controls.

Serum dilutions.	Antisera.			
	Cowpox.	Vaccinia.	Ectromelia.	Variola.
1/10	11	3	11	3
1/50	33	5	14	6
1/250	59	7	14	7
1/1250	74	11	57	15
1/6250	—	56	—	54

TABLE III.—*Neutralization of Ectromelia Virus by Immune Fowl Sera.*

Average number of lesions on chorio-allantois expressed as percentage of controls.

Serum dilutions.	Antisera.			
	Cowpox.	Vaccinia.	Ectromelia.	Variola.
1/10	—	—	3	—
1/50	27	7	4	7
1/250	100	19	15	15
1/1250	93	84	70	22
1/6250	85	90	114	79

TABLE IV.—*Neutralization of Variola Virus by Immune Fowl Sera.*

Average number of lesions on chorio-allantois expressed as percentage of controls.

Serum dilutions.	Antisera.			
	Cowpox.	Vaccinia.	Ectromelia.	Variola.
1/10	34	8	14	1
1/50	117	10	15	3
1/250	96	21	87	5
1/1250	82	66	109	13
1/6250	—	112	—	42

hibited by high dilutions of variola and vaccinial antiserum, moderately well by ectromelia and poorly by cowpox antiserum. Against ectromelia virus the variola antiserum was again the most potent (Table III), vaccinia and ectromelia antisera were rather weaker while cowpox was relatively poor in neutralizing antibody. Against variola virus (Table IV) the order of potency of the sera was variola > vaccinia > ectromelia > cowpox. To obtain a measure of the relative strengths of the antisera, graphs were drawn on log-paper of the results shown in Tables I to IV, the percentage neutralization being plotted against the serum dilutions. The serum dilutions which reduced the number of lesions to 33·3 per cent of the controls are shown in Table V as a measure of serum titres.

It appears from Table V that the cowpox antiserum is relatively weak but specific in its neutralizing activity. The vaccinial antiserum is also relatively

TABLE V.—*Neutralizing Titres of Immune Fowl Sera, Taking as End Point the Dilution of Serum giving 33·3 per cent of the Lesion Count obtained with Normal Serum-Virus Mixture.*

Virus.	Antisera.			
	Cowpox.	Vaccinia.	Ectromelia.	Variola.
Cowpox	660	890	1020	2750
Vaccinia	49	3760	660	3350
Ectromelia	63	470	560	2060
Variola	9	460	102	4470

specific but is much more potent. The ectromelia antiserum neutralizes cowpox rather better than it neutralizes the homologous virus although it is less effective against variola virus. The variola antiserum appears to be the strongest but is not very specific, the titre of antibody for the other three viruses being relatively high.

In previous neutralization tests by the chorio-allantoic inoculation technique these immune fowl sera were tested undiluted but specific differences were not demonstrated (McCarthy and Downie, 1948). It is apparent, however, that tests of the sera in five-fold dilutions provide a better measure of their antibody content and elicit differences not detectable by the use of undiluted serum. Burnet, Keogh and Lush (1937) suggested that differences of neutralizing activity for vaccinia virus between weak and strong rabbit antisera might be more apparent in higher than in lower serum concentrations. However, with our relatively weak immune sera prepared in fowls differences in neutralizing activity against related viruses were more readily demonstrable in higher serum dilutions.

II. *Cross Absorption Experiments.*

The immune fowl sera absorbed with the four viruses, as described under methods, were tested for neutralizing activity in a single concentration. More accurate assessment of the effects of absorption might have been obtained if each absorbed serum had been titrated for neutralizing antibody by the dilution method. This would have entailed a much greater expenditure on materials and it seems doubtful whether the results would have justified the labour involved. The results of neutralization tests with unabsorbed and absorbed sera are shown in Tables VI and VII. The figures again represent the average number of lesion on five or six membranes expressed as a percentage of the average number on

TABLE VI.—*Neutralization Tests with Unabsorbed and Absorbed Sera.*

Average number of lesions on chorio-allantois expressed as percentage of controls.

Antisera.	Infecting virus. Cowpox. Virus used for absorption of sera. None.	Cowpox.	Vaccinia.	Infecting virus. Vaccinia. Virus used for absorption of sera. None.	Cowpox.	Vaccinia.	Infecting virus. Ectromelia. Virus used for absorption of sera. None.	Cowpox.	Vaccinia.	Infecting virus. Variola. Virus used for absorption of sera. None.	Cowpox.	Vaccinia.
Cowpox	10	48	26	5	112	80	6	60	43	2	83	74
Vaccinia	14	54	75	7	9	31	9	6	48	5	15	80
Ectromelia	10	99	28	10	63	44	8	11	5	10	29	37
Variola	20	32	31	5	7	36	8	12	8	4	5	6

TABLE VII.—*Neutralization Tests with Unabsorbed and Absorbed Sera.*

Average number of lesions on chorio-allantois expressed as percentage of controls.

Antisera.	Infecting virus. Cowpox. Virus used for absorption of sera. None.	Ectromelia.	Variola.	Infecting virus. Vaccinia. Virus used for absorption of sera.	Infecting virus. Ectromelia. Virus used for absorption of sera. None.	Ectromelia.	Variola.	Infecting virus. Variola. Virus used for absorption of sera. None.	Ectromelia.	Variola.
Cowpox 1/15	9	60	36		4	65	24	25	103	84
Vaccinia 1/15	15	33	40	Not tested	1	19	14	6	15	91
Ectromelia 1/15	6	82	23		1	101	6	3	124	127
Variola 1/15	13	20	77		7	30	97	5	6	47

membranes receiving the same amount of virus mixed with normal fowl serum. In Table VI are shown the findings in four separate experiments, all sera being tested against one virus at a time ; similarly the results of testing the sera absorb with variola and ectromelia viruses are given in Table VII. The neutralizing activity of the various sera was also tested against alastrim virus, but as the results did not differ materially from those with variola virus they have not been included.

In considering Tables VI and VII the neutralizing power of the unabsorbed sera as shown in Table V should be kept in mind. It may be noted that, in general, absorption of a serum by homologous virus reduced the neutralizing activity of the serum for both homologous and heterologous viruses and that each virus absorbed antibodies for itself from all sera. Quantitative differences in the degree of absorption in different experiments are apparent but these are difficult to analyse because of the variation in antibody content of the individual sera. We have abstracted from Tables VI and VII the figures which show the effect of absorption with the four viruses on the antibody content of each serum for its homologous virus. These are shown in Table VIII, figures in the left half being

TABLE VIII.—*Absorption by Various Viruses of Antibody to Virus used to Prepare the Sera—Data from Tables VI and VII.*

Average number of lesions on chorio-allantois expressed as percentage of controls.

Antisera.	Virus used for absorption of sera.						
	None.	Cowpox.	Vaccinia.		None.	Ectromelia.	Variola.
Cowpox . .	10	*48*	26	.	9	60	36
Vaccinia . .	7	9	*31*	.		not tested	
Ectromelia . .	8	11	5	.	1	*101*	6
Variola . . .	4	5	6	.	5	6	*47*

Figures indicate percentage " survival " of virus homologous to serum before and after absorption.

extracted from Table VI and in the right half from Table VII. The effects of absorption on the antibody content of the sera for their homologous viruses, with the exception of cowpox serum, show that significant reduction was achieved by absorption only with the homologous virus. This suggests a specificity of antibody not demonstrable by neutralization tests with undiluted unabsorbed sera. Cowpox antibody was partially removed from cowpox antiserum by absorption with all four viruses. The effects of absorption with ectromelia and variola viruses are not, however, strictly comparable with those due to absorption with cowpox and vaccinia as the serum absorbed by the first two was diluted 1 in 15 and less virus was used. Moreover, as shown in Table V the titre of the cowpox serum for cowpox virus was less than the titres of antivaccinial and antivariola serum for their homologous viruses.

From the data in Table VI and VII we attempted to analyse the results of reciprocal absorption of pairs of antisera with their respective viruses but in view of the differences in antibody content of the unabsorbed sera it is difficult to interpret the results. However, there is no evidence of complete reciprocal cross absorption between any pair of these viruses and their antisera, and it seems that no two of them are antigenically identical.

DISCUSSION.

When the neutralizing power of immune sera was estimated by testing serum dilutions against constant amounts of virus differences between the sera were detected which had not been shown in tests with undiluted sera (McCarthy and Downie, 1948). With the exception of the ectromelia antiserum each immune serum neutralized the homologous virus to higher titre than it neutralized heterologous virus. Absorption tests confirmed these findings and also demonstrated a difference between ectromelia virus and other members of the group. With the exception of the cowpox serum, which was relatively weak, antibodies to the virus homologous to each serum were considerably reduced only by absorption with that virus.

The close antigenic relationship of ectromelia virus to other members of the group, first observed by Burnet and Boake (1946), has been again confirmed by the present experiments. Downie and Macdonald (1950) using the same immune sera and soluble antigens (LS) from these viruses in inhibition of complement fixation tests found that ectromelia was more closely related to cowpox than it was to vaccinia or smallpox. The same association is shown less clearly by neutralization tests with absorbed and unabsorbed sera. Previous work with vaccinia has shown that the neutralizing power of antivaccinial sera does not run parallel with *in vitro* reactivity with LS antigen (Parker and Rivers, 1936; Salaman, 1937) and therefore the lack of complete correlation of the results of *in vitro* tests involving LS antigens and neutralization tests is not surprising. The immunological differences between cowpox and vaccinia indicated by the present results are in accord with the results of neutralization tests made in rabbits with absorbed sera (Downie, 1939). Differences between vaccinia and variola were not demonstrable by the inhibition of complement fixation test using fowl immune sera and LS antigens from chick embryos or other animal sources (Downie and Macdonald, 1950). The present experiments, however, demonstrate minor antigenic differences between the strains studied in that the titre of neutralizing antibody was higher for the homologous virus; and absorption with vaccinia virus failed to reduce significantly the antibody for variola virus in variola antiserum. The relationship of alastrim to variola virus was not examined in such detail; all the immune fowl sera were, however, tested for neutralizing activity against alastrim before and after absorption with vaccinia and with cowpox virus. Although these viruses removed antibody for alastrim from cowpox, vaccinia and ectromelia immune sera they did not appreciably affect the neutralizing power of variola antiserum for alastrim virus. These findings, as far as they go, do not suggest antigenic difference between the alastrim and the variola strains examined. On the basis of their results in cross immunity experiments in monkeys with the viruses of vaccinia, variola and alastrim, Horgan and Haseeb (1939) put forward the view that vaccinia virus is a degraded antigenic variant of variola and that variola virus possesses, in addition to a basic antigen shared by vaccinia, a specific antigen the presence of which is associated with a high degree of virulence for man. Our own observations suggest that there are minor antigenic differences between the viruses of vaccinia and variola, but whether this antigenic difference determines the infectivity and virulence for different animal hosts is a highly speculative question on which we have no information.

It may be noted that although the techniques used have served to indicate minor antigenic differences in this group these differences are no greater than those observed among different strains of some other viruses, e.g. influenza A. It is known that influenza viruses become altered in their immunological characters on propagation in the laboratory but we have had as yet no convincing evidence of such alteration in the strains of virus under study.

SUMMARY.

A comparative serological study has been made of the viruses of ectromelia, cowpox, vaccinia and variola by means of neutralization tests on the chick chorio-allantois using virus strains propagated in this tissue and immune sera prepared in fowls.

Although cross neutralization tests using undiluted sera had failed to distinguish these viruses from each other, differences were revealed by titrating each serum for neutralizing activity against each virus strain. Differences were also demonstrable by the use of absorbed sera for neutralization tests. There was no complete cross absorption between any pair of these viruses and their antisera and it seems that no two of them are antigenically identical. Our experiments failed to differentiate the viruses of variola and alastrim.

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THE USE OF FILTRATES OF *VIBRIO CHOLERAE* IN THE CLASSIFICATION OF INFLUENZA VIRUS STRAINS.

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SINCE the original isolation of influenza A virus in 1933 there has been considerable evidence to show that this virus is not a single biological entity, but that antigenic differences exist between various strains—particularly between those isolated from different outbreaks. These differences have been demonstrated by neutralization tests *in vivo*, by complement-fixation tests *in vitro*, and by the agglutination-inhibition test of Hirst (1942). In the latter reaction, as shown by Hirst (1942), human and animal sera may yield high titres of inhibition due to non-specific inhibitory substances in addition to those derived from true antiviral antibodies. As a result no firm basis exists for classification of strains by simple titration of immune sera for their power of haemagglutination inhibition *in vitro*, and the results of such titrations may not be confirmed by *in vivo* neutralization tests. Moreover, though amounts of non-specific potentially inhibitory substances in normal sera are themselves variable and unpredictable, the titres found depend to a large extent upon the particular virus used for titration. Francis (1947) considers that the capacity of B strains for combining with non-specific serum inhibitors diminishes on repeated egg passage, but while this may not be a general rule with A strains (Stuart-Harris and Miller, 1947; van der Veen and Mulder, 1950) it seems clear that with any one virus strain this property can be markedly influenced by passage.

It is essential, therefore, in order to increase the precision and the reliability of haemagglutination-inhibition as a means of classifying influenza strains, to find some means of reducing non-specific effects in immune sera. It has been reported by van der Veen and Mulder (1950) that though these can be reduced by treatment of sera by various physical or chemical agents (heat, periodate, removal of calcium ions) these methods are not entirely satisfactory. These authors have found that better results could be obtained by treating serum with crude filtrate of *Vibrio cholerae*, as was first suggested by Mulder and van der Veen (1948). We have been able to confirm this.

METHODS.

Preparation of cholera filtrate.

The medium used for the preparation of cholera filtrate was a semi-solid tryptic digest of heart, without added phosphates, at pH 6·8, as recommended by Mulder (personal communication). This was poured into petri dishes to form a very thin soft layer, and inoculated with approx. 0·2 ml. per plate of an 8-hour broth culture of *V. cholerae* 4Z. After about 16 hours' incubation at 37° C. the soft agar cultures were filtered through filter paper and cotton wool in a Buchner

funnel, and the filtrate clarified, Seitz-filtered, and neutralized. Though the optimal pH for the receptor destroying enzyme (R.D.E.) of this organism has been found by Burnet (1948) to be around 6·0, this was found to be too low for complete elimination of ferret serum inhibitors for influenza A strains, and better results were obtained with a neutral filtrate.

Filtrates were titrated by finding the minimal amount which, with a given amount of normal ferret serum, would eliminate inhibition of virus haemagglutination. Filtrates can be stored at 4° C. for at least six months without loss of activity.

Treatment and Titration of Sera.

Each immune serum to be investigated for antibody level was titrated in duplicate, one portion with added cholera filtrate, and one without, the conditions for both titrations being otherwise identical. For each serum there was thus obtained titres of untreated serum and titres after treatment with filtrate when non-specific inhibition had been eliminated.

The procedure adopted was to add to 0·1 ml. serum (for each virus) either 0·5 ml. calcium saline (0·85 per cent NaCl, 0·1 per cent $CaCl_2$) or 0·5 ml. cholera filtrate appropriately diluted with calcium saline. With each batch of immune sera one normal serum showing a good titre of non-specific inhibition with the particular virus to be used was treated similarly, as an indicator of the satisfactory behaviour of the filtrate. All serum dilutions were incubated at 37° C. overnight, and then heated at 58–59° C. for 50 minutes to inactivate the filtrate. This amount of heating may diminish slightly the level of non-specific inhibition, but does not by any means eliminate it.

The amount of heating required can be decreased if calcium is not incorporated in the diluent (or is subsequently removed by addition of citrate) as the thermal stability of R.D.E. is enhanced by the presence of added calcium (Burnet and Stone, 1947). That there should be no residual active R.D.E. in the titrated sera is important, to obviate destruction of the red cell receptors with resultant loss of virus agglutination which can occur at room temperature during the performance of the titration. Calcium saline was used as diluent in the first experiments because of the reported effect of added calcium upon destruction by R.D.E. of mucopolysaccharide inhibitors (Edney, 1949). In later experiments with crude filtrate it was found that normal saline gave satisfactory results, and addition of calcium was unnecessary for elimination of non-specific inhibitors. The calcium content of the diluent did, however, affect the demonstrable non-specific inhibition of a serum. Thus, as illustrated in Table I, rather higher titres were found in normal sera diluted initially with calcium saline, even though the rest of the titrations were carried out with normal saline.

Virus strains.—Influenza A strains were represented by N-W.S. (a neurotropic variant of W.S. (Stuart-Harris, 1939)); PR8—the classical influenza A strain isolated in 1934; Kunz—isolated during a local outbreak in this country in 1947 (Stuart-Harris and Miller, 1947); NED/1/49—a strain from Holland isolated in 1949 and found by Chu, Andrewes and Gledhill (1950), to belong to the so-called influenza A prime group; and a number of other strains isolated locally during the outbreak of 1949, whose origin is described elsewhere (Stuart-Harris, Laird, Tyrrell, Kelsall, Franks and Pownall, 1949).

TABLE I.—*Effect of Calcium upon the Inhibitory Titres of Normal Ferret Sera for Virus Haemagglutination.*

Virus.	Normal ferret serum.	Serum diluent.	Incubation 37° C.	Heating.	Agglutination at serum dilutions of						
					1/24	1/48	1/96	1/192	1/384	1/768	1/1536
KUNZ	1	N.S.	18 hrs.	59° C., 50 min.	0	0	0	0	+++	+++	
		Ca.S.	,,	,, ,,	0	0	0	0	0	++	+++
		N.S.	None	None	0	0	0	0	++	+++	
		Ca.S.	,,	,,	0	0	0	0	0	+++	
KUNZ	2	N.S.	18 hrs.	59° C., 50 min.	0	0	0	+	+++	+++	+++
		Ca.S.	,,	,, ,,	0	0	0	0	0	++	+++
		N.S.	None	None	0	0	0	0	++	+++	+++
		Ca.S.	,,	,,	0	0	0	0	0	++	+++
NED	3	N.S.	18 hrs.	59° C., 50 min.	0	0	+++				
		Ca.S.	,,	,, ,,	0	0	0	0	+++		

N.S. = Normal saline. Ca.S. = Calcium saline.

(After the initial serum solution (0·2 ml. serum + 1·0 ml. diluent) the diluting fluid was in all cases N.S.)

All viruses were egg-adapted, used either in the form of pooled allantoic fluids or of concentrates prepared from these by elution from erythrocytes.

Immune sera.—Convalescent sera were obtained from ferrets by cardiac puncture 10 to 14 days after intranasal inoculation. In some cases a serum sample obtained from the same ferrets before inoculation was available for comparison (pre-infection sample).

Method of titration.—Titrations were carried out by a method similar to that described by Salk (1944), using fowl cells and (with a few exceptions) 8 haemagglutinating doses of virus.

RESULTS.

Results of cross titrations, both with and without the use of cholera filtrate, are presented in Table II. Inhibition titres demonstrated diagrammatically in Fig. 1 represent the results with cholera-treated sera only.

It is evident that crude filtrate can, under the conditions of these experiments, eliminate all non-specific inhibition of red cell agglutination. The findings also show that, when non-specific effects were eliminated, the influenza strains which were examined fell into three distinct antigenic groups with little apparent cross-reaction: W.S., PR8, and the group whose representatives here appeared in 1947 and 1949, and which is probably the same as that termed by American authors the influenza A prime group.

Of the latter group it would appear that some strains, notably Shef./10/49 and Shef./8/49, resemble the "Q strains" of Mulder, van der Veen, Brans and Enserink (1949) in that, though not poor antibody producers, they will only detect low levels of antibodies in homologous and heterologous sera. A glance at the titres obtained with the same virus strain with different sera, all of which exhibit some residual inhibition shows, however, the impossibility of drawing conclusions in regard to the degree of antigenic overlap from the quantitative level of inhibition obtained in this modified inhibition test. For example, the 1947 KUNZ virus gave high titres of inhibition with many of the sera from 1949 strains, yet the KUNZ antiserum was relatively feeble in its action on the 1949 strains compared with its titre for homologous virus.

Because so many variable factors are involved in the inhibition test it seems improbable that the method will reveal fine differences between related strains until greater precision and standardization can be achieved.

Treatment of Sera in Parallel with Crude Filtrate and with R.D.E.

A preparation of "purified" R.D.E. was obtained by the courtesy of Professor F. M. Burnet, and compared with crude cholera filtrate for its capacity to remove non-specific inhibition for influenza A strains from normal ferret sera. Results obtained with two sera, titrated with a virus sensitive to non-specific inhibition, are shown in Table III. It is quite evident that while the filtrate destroyed in each case all non-specific inhibition of haemagglutination the enzyme preparation reduced it only slightly. That the higher titre in the latter cases was not due to destruction of red cell virus receptors by residual R.D.E. is shown by the fact that no titre was obtained in the absence of serum.

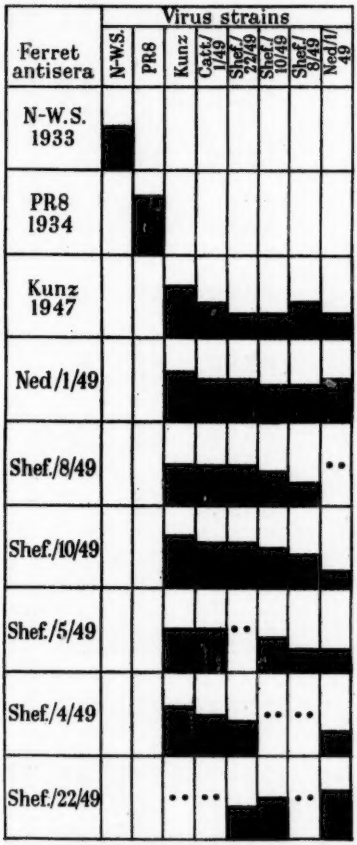

FIG. 1.—Antibody titres of ferret sera, treated with cholera filtrate, for homologous and heterologous strains of virus. The shaded areas represent logarithms of titre increases as multiples, where the reciprocal of the lowest dilution (1/24) is taken as unity. Actual titres are given in Table II. Titres with NED virus were sometimes difficult to interpret owing to rapid elution. .. = No test. Titres with PR8 virus and NWS sera were variable but some inhibition was obtained with three of four sera tested.

Investigation of Influenza B Strains.

As it became clear that the recently isolated influenza A strains examined here showed little antigenic relationship in haemagglutination inhibition titrations to the early strains of 16 years ago it was decided to investigate a limited number of influenza B strains in order to determine whether a similar deviation from the parent type had occurred amongst this group.

The methods of preparation and titration of ferret convalescent sera were the same as for influenza A strains. Viruses representative of the B group were LEE 1940 and two strains isolated locally from a school outbreak of influenza during the early months of 1950—TOD and COW. Acute and convalescent

TABLE II.—*Titres of Ferret Antisera, with and without Treatment with* V. cholerae *Filtrate, for Homologous and Heterologous Viruses.*

	Virus strains.															
	N-W.S.		PR8		KUNZ		CATT/1/49		SHEF./22/49		SHEF./10/49		SHEF./8/49		NED/1/49	
Treatment with cholera filtrate	—	+	—	+	—	+	—	+	—	+	—	+	—	+	—	+
LEE B strain	768	<24	*<24*	*<24*	*192*	*<24*	384	<24								
N-W.S. 1933																
Pre-inf.	48	<24	*<24*	*<24*	*320*	*<24*										
Post-inf.	3072	1536	*160*	*160*	*768*	*<24*	192	<24	192	<24	96	<24	<24	24	48	<24
PR8 1934	1536	<24	*1536*	*1536*	*768*	*<24*	384	<24	384	<24	192	<24	24	24	192	<24
KUNZ 1947	768	<24	*<24*	*<24*	*1536*	*768*	192	96	192	48	192	48	<96	96	192	96[a]
NED/1/49	1536	<24	*<24*	*<24*	*768*	*384*	384	192	384	192	192	96	96	96	768	384[a]
SHEF./9/49	1536	<24	*<24*	*<24*	*1536*	*384*	384	96	384	192						
SHEF./7/49	768	<24	*<24*	*<24*	*1536*	*768*	768	384							384	96
SHEF./8/49	768	<24	*<24*	*<24*	*768*	*192*	768	192	768	192	192	96	96	48		
SHEF./10/49	768	<24	*<24*	*<24*	*1536*	*768*	768	384	384	384	384	192	96	96	48	48
SHEF./18/49	192	<24	*<24*	*<24*	*1536*	*384*	768	192								
SHEF./1/49	192	<24	*<24*	*<24*	*768*	*192*	192	48							96	24
SHEF./5/49	192	<24	*<24*	*<24*	*1536*	*192*	384	192			384	96	96	48	48	48
SHEF./4/49	96	<24	*<24*	*<24*	*1536*	*384*	384	192	384	96					192	48
SHEF./6/49																
Pre-inf.	192	<24	*<24*	*<24*	*384*	*<24*									**48**	**<24**
Post-inf.	192	<24	*<24*	*<24*	*1536*	*768*	768	768							**96**	**48**
SHEF./22/49																
Pre-inf.	48	<24	*<24*	*<24*					<24	<24						
Post-inf.	48	<24	*<24*	*<24*					96	96	384	192			768	768[a]
Normal ferrets	48	<24			*768*	*24*	384	<24	<24	<24	192	<24	24	<24	192	<24

(a) = 4 haemagglutinating doses of virus. Pre-infection serum was obtained immediately before inoculation. Post-infection serum 10–14 days after inoculation. Figures in italics are titres obtained with virus in the form of an eluate; in all other cases the virus preparation consisted of pooled egg-allantoic fluids. (Results with NED virus were sometimes difficult to interpret owing to the great readiness with which this virus elutes from red cells at room temperature.)

TABLE III.—*Inhibitor Titres of Normal Ferret Sera Treated with "Purified" R.D.E. and with Cholera Filtrate.*

Serum.	Components—incubated and heated.	Haemagglutination at dilutions						
		1/24	1/48	1/96	1/192	1/384	1/768	1/1536	1/3072
Normal ferret (24)	0·2 ml. serum + 1·0 ml. Ca. saline	0	0	0	0	0	0	±	+++
	0·2 „ + 1·0 ml. filtrate[1]	+++	+++						
	0·2 „ + 1·0 ml. R.D.E.[2]	0	0	0	0	0	++	+++	+++
Normal ferret (15)	0·2 ml. serum + 1·0 ml. Ca. saline	0	0	0	0	0	0	+++	+++
	0·2 „ + 1·0 ml. filtrate[1]	+++	+++						
	0·2 „ + 1·0 ml. R.D.E.[2]	0	0	0	0	0	+++	+++	
None	0·2 „ + 1·0 ml. R.D.E.[2]	+++	+++						

Virus = KUNZ ; 4 H.D.

(1) An equivalent amount of calcium added.

(2) In solution in calcium acetate buffer, containing 200 units R.D.E./ml.

serum samples from the two patients from whom the latter viruses were recovered had already been found, by complement fixation test, to show a significant rise in antibodies to influenza B virus. However, in haemagglutination inhibition tests using LEE virus, while COW sera showed a parallel rise in inhibitory titre from 20 to 160, TOD sera did not inhibit haemagglutination at all.

For titrations of ferret sera all viruses were used in the form of pooled infected egg allantoic fluids.

Results of cross titrations are presented in Table IV. It is apparent that as two of the viruses concerned are affected by non-specific serum inhibitors it is only from results with cholera filtrate treated sera that their relationships are clarified. Thus it can be seen from Table V that the recently isolated strains of influenza B differ immunologically from the original parent LEE, but resemble each other closely.

TABLE IV.—*Titres of Ferret Antisera, with and without Treatment with* V. cholerae *Filtrate, for Homologous and Heterologous B Viruses.*

		Virus strains					
		LEE		TOD		COW	
Ferret Antisera.	Treatment with cholera filtrate	−	+	−	+	−	+
	LEE	768	768	96	<24	192	<24
	TOD Pre-inf.	—	—	192	<24	—	—
	Post-inf.	24	24	768	768	768	768
	COW Pre-inf.	—	—	—	—	384	<24
	Post-inf.	24	24	768	384	768	384
	Normal ferrets	<24	<24	96	<24	192	<24

TABLE V.—*Titres of Antisera Treated with* V. cholerae *Filtrate for B Viruses.*

	Virus strains.		
Ferret Antisera.	LEE	TOD	COW
LEE	768	0	0
TOD	24	768	768
COW	24	384	384

DISCUSSION.

Since the discovery by Francis (1947) that a component of normal serum can inhibit haemagglutination by heated LEE virus, much attention has been focused upon the properties of the "Francis inhibitor" (found to be a muco-polysaccharide) and its related red cell virus receptors, both of which are a substrate for influenza virus and for R.D.E. The Francis inhibitor can therefore be eliminated by treatment of serum with this enzyme. Less is known about a second component of normal sera—the "non-specific inhibitor"—which prevents haemagglutination by living influenza A and B strains, and which possesses quite different properties from the Francis inhibitor. Some of the differences have been investigated by McCrea (1948). The non-specific inhibitor, associated with the γ-globulin fraction of serum, is not noticeably changed by R.D.E. or by periodate, but is more heat labile.

"Purified" R.D.E. alone, therefore, is not adequate as a tool for eliminating

non-specific inhibition of haemagglutination by influenza strains. There is present in cholera filtrate a second unidentified substance which is essential, and the conditions required are not precisely those prescribed by other workers for the optimum activity of R.D.E. Nor can a true estimate of the potency of a filtrate be made by titrating the red cell receptor-destroying capacity, as described by Burnet and Stone (1947) for R.D.E.

The question of antibody destruction by enzymes of the filtrate remains uncertain. Though Wilson Smith and Westwood (1948) have found crude cholera filtrates to have too high a content of proteinase to be practicable for use with immune serum, this may well vary with different strains of *V. cholerae*, and with the conditions of cultivation. In fact, during the present investigation, two indications were found suggesting that the antibody loss is not sufficient to be detected by this method of titration: Firstly, the PR8 and LEE strains were not capable of detecting non-specific inhibitors in any serum tested, and in titrations of homologous ferret sera with these viruses no reduction in titre was ever found as the result of filtrate treatment; and secondly, immune hamster sera, which were not demonstrated to contain non-specific inhibitory substances, showed no reduction in homologous titre after contact with filtrate, although normal ferret sera titrated in parallel showed no residual inhibition of haemagglutination after treatment with cholera filtrate.

SUMMARY.

1. By treating immune ferret sera with crude filtrates of *V. cholerae* 4Z under specified conditions all demonstrable non-specific inhibitors of virus haemagglutination were removed. This required the presence in filtrates of an unidentified substance other than receptor-destroying enzyme. It would not appear that there was much, if any, concomitant loss of antibody.

2. The findings of van der Veen and Mulder (1950) were confirmed in that, when non-specific titres were eliminated, influenza A and B strains could be broadly classified by the haemagglutination test using convalescent ferret sera.

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THE EFFECT OF RENAL ARTERY CONSTRICTION ON THE RENAL BLOOD FLOW.

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A SERIES of experiments have been carried out in which the blood supply to one kidney has been reduced, and the resulting changes in the intra-renal distribution of the circulating blood studied by intra-arterial injection of dyes.

METHOD.

In 14 rabbits anaesthetized with nembutal and ether, the left renal artery was exposed through a lumbar incision and constricted either by a ligature to $\frac{32}{1000}$ or $\frac{38}{1000}$ inches described by Drury (1938), or by a silver clip of $\frac{16}{1000}$ or $\frac{20}{1000}$ inch by the method of Pickering and Printzmetal (1938). This degree of contriction is somewhat greater than is necessary to produce hypertension, which in any case is unlikely to develop with an intact kidney present on the other side. After an interval varying from three days to five weeks, intra-arterial injections of 2 per cent berlin blue or of 33 per cent Higgins indian ink in distilled water were made in the anaesthetized or recently killed animal. In the live animal retrograde injections were made through a cannula inserted into the abdominal aorta below the origin of the renal arteries and pointing centrally, and in the dead through a cannula inserted into the renal artery proximal to the constriction. The results obtained by the two methods were the same although the concentration of dye was less in the *in vivo* injections, and consequently it was not possible to follow the course of the smaller vessels with the same exactitude as after post-mortem injection. The injections were continued until the dye was seen emerging freely from the renal vein. The kidney was then rapidly removed to formalin. In the animals which had retrograde aortic injections the normal kidney was also removed, and was used as a control to ensure that necessary intra-abdominal manipulations had not disturbed the renal circulation. Two types of section were prepared, frozen sections 200μ thick to show the distribution of the dye, and paraffin sections 5μ thick stained with haematoxylin and eosin for histological examination.

RESULTS.

In every case the deepest layer of the cortex—including the juxta-medullary zone of Trueta and his colleagues—contained dye. This could be seen clearly both on the arterial and venous sides (Fig. 2*a*). A varying thickness of the superficial cortex was completely free from dye (Fig. 1 and 2*a*).

Histological examination of the kidneys showed a difference between the

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† With a grant from the Medical Research Council.

superficial and the deeper zones of the cortex. In some kidneys (Fig. 1) there was complete necrosis of the superficial zone of the cortex and of the medulla, but not of the deeper cortical zone. The necrotic areas did not stain at all with haematoxylin and eosin. The deeper, dye-containing zone of the cortex in these kidneys stained well : this deep zone showed signs of degeneration with pyknotic changes in the nuclei, and collections of cellular debris, but the changes were considerably less than in the superficial dye-less zone.

In other kidneys (Fig. 2*a*) the superficial zone, which had not filled with dye, showed early degenerative changes. The glomeruli appeared to have shrunk in their Bowman's capsules and there was an increase in the cellularity. An occasional polymorph was present in their glomerular tufts. The proximal convoluted tubules showed degeneration of their epithelium, and some cells had desquamated into the lumen. The deeper zone of the cortex did not show these changes. Here the glomeruli were not shrunken, there was no appreciable change in their cellularity, and the proximal convoluted tubules showed only very slight changes.

DISCUSSION.

The course taken by the dye injected into the blood stream *in vivo* must be the same as that taken by the blood. From the results of the dye injection and of histological examination it is clear that the circulation through the peripheral nephrons had been reduced to a much greater extent than that through the more central ones.

It is improbable that the anastomotic vessels to the kidney are responsible for their changes. The capsular vessels would, if anything, tend to increase the blood supply to the more superficial glomeruli. The ureteric vessels would, for hydrodynamic grounds, be unable to influence the distribution of the blood from the interlobular arteries to the glomerular arterioles, unless they anastomosed with the renal vessels at the level of the interlobular arteries, or even further distally. While the point was not specifically investigated in this series of experiments, there is no evidence that such a state of affairs, which would in fact mean a second renal circulation, exists (Trueta, Barclay, Daniel, Franklin and Prichard, 1947).

It is unlikely that this is due to the effects of interference with renal plexus of nerves ; the results are too constant to be the result of neurogenic arterial spasm. Nor does it appear to be connected with the tubular obstruction of capillaries (T.O.C.) of Hartcroft (1948), which produces an ischaemia of quite different distribution.

The findings cannot be explained on hydrodynamic grounds. Trueta and his colleagues (1947) divided glomeruli into juxta-medullary and cortical types on the basis of their morphological structure rather than of anatomical position. However, both in our experiments and many of those illustrated by Trueta and his colleagues, the blood appears to be flowing through the deeper layers of the cortical glomeruli as well as through the narrow zone of juxta-medullary glomeruli. Differences in the resistance offered by the two types of glomeruli cannot therefore be the whole of the explanation of these findings.

Our findings might be explained by an active constriction of the vessels supplying the distal glomeruli, thus maintaining the blood flow to the proximal glomeruli, possibly as a reflex effect of oxygen lack. In this way the circulation could be maintained at a normal level to the more central nephrons throughout wide

variations in blood pressure and renal blood flow, while the amount of blood reaching the more peripheral nephrons might be sufficient to maintain the life of the cells, but insufficient for glomerular filtration.

If this was the case, it would explain the results of Van Slyke and his colleague (1948), who concluded from clearance studies that in haemorrhagic shock " some nephrons were being totally deprived of their blood supply, while the remainder continue to be perfused in the normal manner "; and of Corcoran and Page (1943), who obtained similar results in dogs in which the renal blood flow was reduced by bleeding.

A progressive constriction of the peripheral vessels of this type with maintenance of the existing circulation through the deeper glomeruli, (rather than an active diversion of blood from one part of the kidney to another) may be the basis of the phenomena described by Trueta and his colleagues (1947). If this is so, much of the criticism of this work on the grounds that no qualitative changes can be demonstrated by clearance methods is no longer valid. With a proportion of the nephrons functioning normally while the remainder are not functioning at all, quantitative but not qualitative changes would be expected.

Finally the possibility exists that prolonged vaso-constriction of this type may be responsible for the ischaemia which is held to be partly responsible for the " renal anoxia " syndrome (Maegraith, Havard and Parson, 1945).

SUMMARY.

The renal blood flow in rabbits was reduced on one side by the application of a ligature or silver clip.

Intra-arterial injections of dyes and histological examination showed that blood supply to the deeper cortical region had been maintained to a much greater extent than that to the superficial cortex.

The implications of this finding are discussed.

Our thanks are due to Dr. P. M. Daniel and Miss M. M. L. Prichard for advice about the techniques employed, and to Dr. G. S. Dawes and Dr. A. H. T. Robb-Smith for advice and encouragement.

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EXPLANATION OF PLATE.

Fig. 1.—Photograph of kidney after *in vivo* injection of 33 per cent indian ink 14 days after renal artery constriction. The ink has reached only the juxta-medullary zone.

Fig. 2*a*.—Shadowgraph of section of kidney after post-mortem injection of 2 per cent berlin blue 11 days after constriction of renal artery. The dye has entered the proximal glomeruli, arteries and veins, but not in the more distal vessels.

Fig. 2*b*.—Normal kidney section for comparison.

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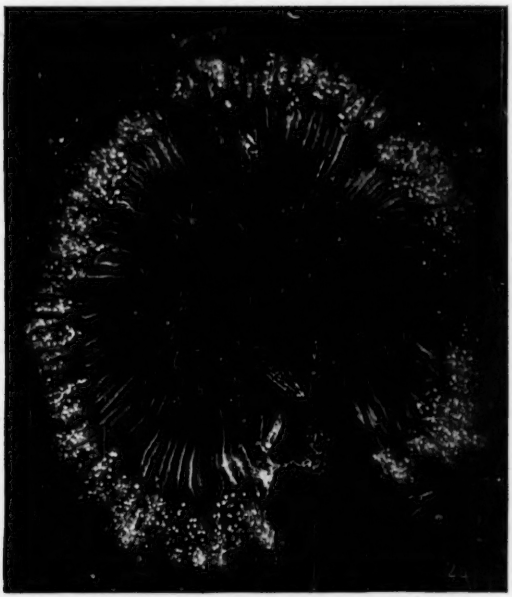

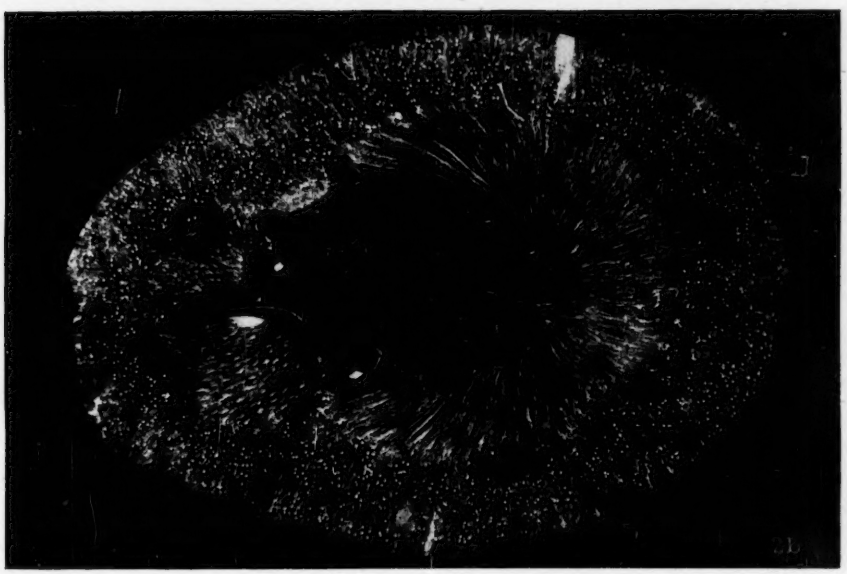

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THE SIZE OF COXSACKIE VIRUSES AS ESTIMATED BY FILTRATION THROUGH GRADOCOL MEMBRANES.

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In view of the many similarities between poliomyelitis and Coxsackie viruses, and in particular their simultaneous appearance in human stools, sewage, and flies, it appeared to be desirable to have exact measurements of the size of Coxsackie viruses.

Previous observations by Quigley (1949), working with strain "T.T." ("Dalldorf I" type), suggested that the virus passed through an 18 mμ. membrane; the size of the virus would therefore be 10 mμ. or less. Melnick (1950), without giving exact figures, considered that the diameter of different immunological types was of the order of 15 to 23 mμ.

MATERIAL AND METHODS.

Filtration studies were carried out with two strains of Coxsackie virus which serologically are unrelated. They both produce paralysis in suckling mice. Strain "High Point" (North Carolina) was isolated from urban sewage during an epidemic of poliomyelitis in 1948 (Melnick, 1950). "Dalldorf I" strain was differentiated from other Coxsackie viruses by Sickles and Dalldorf (1949). The infective titres of a 10 per cent brain emulsion were 10^{-5} or 10^{-6} for "High Point" and 10^{-4} or 10^{-5} for "Dalldorf I." After intraperitoneal inoculation of a 10 per cent suspension of infected muscle the incubation period was slightly shorter for "High Point" than for "Dalldorf I" as shown in Table I. No significant differences in clinical symptoms were noted.

TABLE I.—*Incubation Period of Coxsackie Viruses in Mice.*

Strain.	Number of mice inoculated.	Number of mice showing paralysis in hours after inoculation. Hours.					Average incubation period (hours).
		30	48	54	72	96	
"High Point"	184	8	67	50	29	0	48·4
"Dalldorf I"	63	24	11	2	15	11	55·4

Preparation of stock virus suspensions.

Freshly prepared 10 per cent (w/v) brain emulsions in digest broth (brains from suckling mice, infected intraperitoneally with either strain, and harvested when the mice were paralysed) were centrifuged for 30 minutes at 3000 r.p.m. The supernatants were then again centrifuged for 45 minutes at 18,000 r.p.m. (25,000 g.) by the aid of the Multispeed Attachment of an International Equipment Co. Centrifuge, Size 2, in a cold room (+6° C.). The temperature of the

second supernatants rose to 35° C., but little or no loss of titre was observed. Further clarification was not required, for it was found that appreciable amounts of the second supernatants (stock suspensions) passed readily through gradocol membranes.

Gradocol membranes and filtration technique.

The membranes were prepared at the Wright-Fleming Institute in accordance with the methods described by Elford (1938). The filtration pressure never exceeded 1 atmosphere, and the volumes filtered per 1 sq. cm. were for primary filtrates 3 to 4 ml. and 5 to 10 ml. for secondary or tertiary filtrates.

Testing of suspensions and filtrates.

Quantities of 0·05 ml. of the undiluted or serially ten-fold diluted stock suspensions or filtrates were injected intraperitoneally into 3 to 6 (4 to 5 day old) mice. The titration end-point was taken to correspond to the dilution which produced death or paralysis in at least one mouse within 7 days.

Testing of sera for presence of antibodies.

In some experiments surviving mice were bled 2 to 3 weeks after receiving an inoculum, and the sera tested for the presence of neutralizing antibodies. The undiluted sera were mixed with an equal volume of increasing dilutions of a virus suspension, and were left for 2 hours on the bench before being injected into 4 to 5 day old mice. Sera from normal mice were treated similarly to act as a control.

FILTRATION STUDIES.

Strain "High Point."

In preliminary experiments with stock suspensions it was established that the virus passed without loss through a membrane of an average pore diameter (A.P.D.) of approximately 120 mμ. It was also found that decreasing amounts of the virus passed through membranes of progressively lower porosities down to 36 mμ., but that a membrane of an A.P.D. of 31 mμ. retained it (Table II). Owing, however, to the presence of particulate non-viral material, the volume passing through these membranes was less than 5 ml./sq. cm. Further studies were therefore conducted with preparations which had passed through membranes of varying porosity. The yields now obtained were of the order of 5 to 10 ml./sq. cm. and, as can be seen from Table II, the relative amount of virus passing through membranes of the same A.P.D. was larger for the preparations which had already been filtered. This is in agreement with the observations of Elford (1938), who recommended, for the exploration of the filtration end-point range, the use of filtrates which had passed through a membrane of the lowest A.P.D. which just does not retain the virus.

In employing finally a stock suspension (Batch No. X) which had been filtered first in succession through membranes of 110 mμ. and 41 mμ. A.P.D. respectively, we found that, although the titre had dropped to 10^{-2}, the virus now passed through a membrane of 32 mμ. but not through a membrane of 28 mμ.

The end-point of filtration is therefore considered to be 30 mμ. for the "High Point" strain of Coxsackie virus and its probable particle size 10 to 15 mμ.

Strain "Dalldorf I."

The results of the filtration experiments with this strain are summarized in Table III. The virus passed through all membranes having a greater A.P.D.

TABLE II.—*Filtration of Coxsackie Virus (Strain "High Point").*

Number of brain emulsion used for preparation of suspension.	A.P.D. in mμ. of membrane for previous filtration.	Titre (A) of suspension or previous filtrate.	A.P.D. in mμ. of membrane for present filtration.	Titre (B) of present filtrate.	Titre (B) / Titre (A) × 100.
I	730	10^{-4}	210	10^{-4}	100
VI	..	10^{-5}	120	10^{-5}	100
V	..	10^{-5}	120	10^{-4}	10
VII	..	10^{-5}	110	10^{-4}	10
IX	..	10^{-6}	110	10^{-5}	10
X	..	10^{-6}	110	10^{-5}	10
II	730	10^{-4}	83	10^{-3}	10
VIII	680	10^{-4}	83	10^{-3}	10
II	730	10^{-4}	60	10^{-3}	10
III	180	10^{-4}	45	10^{-2}	1
IV	..	10^{-5}	41	10^{-1}	0·01
VI	..	10^{-5}	41	10^{-1}	0·01
VI	120	10^{-5}	41	10^{-3}	1
X	110	10^{-5}	41	10^{-2}	0·1
VII	..	10^{-5}	36	10^{-1}	0·01
VII	110	10^{-4}	36	10^{-2}	1
IX	110	10^{-5}	36	10^{-1}	0·01
X	41	10^{-2}	32	10°*	1
IV	..	10^{-5}	31	0†	0
V	..	10^{-5}	31	0†	0
III	180	10^{-4}	31	0†	0
V	120	10^{-4}	31	0†	0
IX	110	10^{-5}	28	0†	0
VIII	83	10^{-3}	28	0†	0
X	41	10^{-2}	28	0†	0

* 3 out of 6 inoculated mice, all of which were paralysed, recovered. Their pooled sera were negative for neutralizing antibodies.

† All mice employed in this test were free of signs of paralysis, and no neutralizing antibodies were detected in their pooled sera.

.. = No previous filtration.

than 32 mμ. Since the filtrates employed for the investigation of the A.P.D. range just above 32 mμ. were similar to those used in the corresponding experiments of the previous series, and the relative amount of virus passing through the membranes was of the same order, it is concluded that the filtration end-point for the "Dalldorf I" strain of Coxsackie virus is identical with that for "High Point," and its probable particle size is therefore also 10 to 15 mμ.

DISCUSSION.

The observations here recorded show that the particles of both the strains of Coxsackie virus examined are similar in size, 10 to 15 mμ. The size of poliomyelitis virus is estimated by Theiler and Bauer (1934) as 12 to 17 mμ., and by Elford, Galloway and Perdrau (1935) as 8 to 12 mμ.; the latter finding is con-

TABLE III.—*Filtration of Coxsackie Virus (Strain " Dalldorf I ").*

Number of brain emulsion used for preparation of suspension.	A.P.D. in mμ. of membrane for previous filtration.	Titre (A) of suspension or previous filtrate.	A.P.D. in mμ. of membrane for present filtration.	Titre (B) of present filtrate.	$\frac{\text{Titre (B)}}{\text{Titre (A)}} \times 100$.
I	..	10^{-4}	170	10^{-4}	100
II	..	10^{-4}	170	10^{-4}	100
III	..	10^{-4}	150	10^{-4}	100
I	170	10^{-4}	41	10^{-2}	1
I	170	10^{-4}	36	10^{0}	0·01
II	170	10^{-4}	36	10^{0}	0·01
III	150	10^{-4}	36	10^{-1}	0·1
I	170	10^{-4}	32	0	0
III	150	10^{-4}	32	0	0
I	170	10^{-4}	28	0	0
II	170	10^{-4}	28	0	0
III	150	10^{-4}	28	0	0

.. = No previous filtration.

firmed by Kling, Olin and Gard (1938). It appears, therefore, that to the many features which the Coxsackie and poliomyelitis viruses have in common, their similarity in size can be added.

Using also the filtration method, Sanders and Jungeblut (1942) reported that the Columbia SK virus, one of the encephalo-myocarditis group, is from 10 to 15 mμ. This virus is therefore the same size as the Coxsackie viruses here studied, but the Coxsackie viruses, apart from this similarity, are quite distinct from the viruses of the encephalo-myocarditis group.

SUMMARY.

1. A method, involving high-speed centrifugalization, for the production of clarified suspensions of Coxsackie viruses from infective mouse brain is described.

2. By the aid of ultrafiltration analysis of such suspensions, the particle size of two serologically unrelated strains of Coxsackie virus has been estimated to be 10 to 15 mμ.

Our thanks are due to Professor Sven Gard for the " High Point " strain, to Dr. F. O. MacCallum and Dr. J. A. Dudgeon for the " Dalldorf I " strain of Coxsackie virus; and to Dr. W. J. Elford, F.R.S., who has given us valuable advice on filtration. The inoculation experiments were carried out in the laboratories of Professor G. A. H. Buttle, to whom we desire to express our gratitude. One of us (G.M.F.) is in receipt of an expenses grant from the Medical Research Council.

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THE EFFECTS OF HUMAN PITUITARY GONADOTROPHINS ON HYPOPHYSECTOMIZED RATS.

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THE kaolin-adsorption method as modified for concentrating urinary gonadotrophins in the absence of pregnancy (Dekanski, 1949*a*) yields crude concentrates which may contain other hormones from both the anterior and posterior pituitary. It is possible also that the results previously obtained on intact immature animals were complicated by the release of hormones by the animals' own pituitary gland. It was decided, therefore, to investigate some of the other effects of the crude gonadotrophic concentrates, and also to carry out the gonadotrophic experiments on immature hypophysectomized animals.

METHODS.

Tests were carried out for oxytocic, pressor, adrenocorticotrophic and histaminic effects.

For gonadotrophic effect only hypophysectomized immature 21–22-day-old albino rats, fed on an Aberdeen standard diet, were used. For the purposes of the experiment, no attention was paid to the litters. The extracts were injected subcutaneously after a 48-hour post-operative interval, and tested pharmacologically and morphologically for their primary and secondary effects as previously described. The pituitary gland was removed by the retropharyngeal approach, the sella turcica of each animal being examined at the necropsy for the completeness of hypophysectomy. The oxytocic activity was determined on the virgin guinea-pig's uterus (Dale and Laidlaw, 1912), suspended in Ringer solution, but with no Mg. in the bath (Burn, 1937), Pitocin (Parke, Davis and Co.) being used as the standard preparation. The pressor activity was assayed on rats' blood pressure (Landgrebe, Macaulay and Waring, 1946), Pitressin (Parke, Davis and Co.) being used as the standard preparation. The adrenocorticotrophic test was carried out by the method depending on adrenal ascorbic acid response in hypophysectomized rats (Sayers and Sayers, 1946). The extraction, estimation and identification of histamine was carried out by the method of Barsoum and Gaddum (1935) as modified by Code (1937). The finely powdered concentrates were ground in a mortar, and extracted for 1 hour with 10 per cent trichloroacetic acid, using 10 c.c. of acid to 300–400 mg. of material. After filtering by gravity, an aliquot of 5 c.c. of filtrate was taken and heated with 5 c.c. of conc. HCl for 90 minutes, on a boiling water-bath. The liquid was then evaporated to 5 c.c. *in vacuo*, and the rest of the acid and water removed by washing the flask down twice with absolute alcohol, and taking to dryness *in vacuo* each time. The extracts were tested on a piece of guinea-pig's ileum suspended in 2 c.c. of Tyrode's solution containing atropine (0·1 μg./c.c.) in

comparison with a standard solution of histamine acid phosphate (The British Drug Houses, Ltd.). At the end of each experiment a small amount of neoantergan (pyranisamine maleate, May and Baker, Ltd.) was added to the bath, the rest of the procedure being as previously described (Dekanski, 1949*b*). Concentrations of histamine present were calculated in terms of histamine base.

RESULTS.

Effect of crude urinary gonadotrophins on the reproductive organs in hypophysectomized immature rats.

The individual results of these experiments are given in Table I. The microscopic changes are shown in Fig. 1 to 6. The data obtained show that the actual

TABLE I.—*Individual Responses of Reproductive Organs in Hypophysectomized Immature Rats to Crude Urinary Gonadotrophins* (≡ 200 *c.c. of Urine*). *Total Individual Dose* ≡ 1·0–1·5 *c.c.*

Subject, Age, Sex. (M) = Male (F) = Female.	Rat No.	Necropsy: weight of			Rat No.	Necropsy: weight of			
		Body (g.).	Uterus (mg.).	Ovaries (mg.).		Body (g..)	Testes (mg.).	Prostate (mg.).	Seminal vesicles (mg.),
	1	41	11·7	7·1	26	38	93	8·0	4·8
	2	53	11·5	7·0	27	46	104	12·0	5·2
	3	53	12·0	7·3	28	50	110	15·6	5·9
	4	45	12·4	6·7	29	48	127	11·8	5·7
					30	42	102	8·4	5·8
D 68 (F) .	6	47	79·0	37·8	31	43	250	39·2	8·8
	7	47	80·0	32·8	32	38	263	42·1	9·9
	8	43	82·0	40·4	33	43	301	38·9	9·2
	9	47	79·0	40·0	34	42	272	37·8	8·0
	10	50	75·0	39·7	35	48	269	38·7	8·9
D 52 (M) .	11	51	45·4	10·8	36	50	325	46·0	6·5
	12	50	42·0	11·2	37	48	321	47·6	7·8
	13	47	33·1	12·3	38	50	340	40·2	7·2
	13	43	36·2	9·8	39	49	352	48·0	7·2
	15	40	40·0	10·9	40	52	363	48·1	8·2
B 26 (M) .	16	42	30·0	11·8	41	50	180	35·0	9·4
	17	43	21·7	9·0	42	52	200	33·9	7·5
	18	49	25·0	9·2	43	47	140	28·8	7·8
	19	50	32·0	10·3					
	20	48	30·2	11·0					
B 23 (F)* .	21	53	22·1	10·6	46	40	104	8·1	4·6
	22	48	20·4	9·0	47	48	140	12·0	5·5
	23	47	11·4	5·5	48	42	70	8·9	5·0
	24	50	12·9	6·3	49	50	89	12·6	6·8
					50	47	110	8·9	6·1

* Nullipara with regular menstrual cycle. The samples were collected on the 18th and 19th day after the beginning of menses.

increase in the weight of reproductive organs of immature hypophysectomized rats in general appears to be similar to that seen in immature intact rats when given the same dose (equivalent to 200 c.c. of urine) of crude urinary gonadotrophins for the same period of time. In the female rats the response to post-

menopausal concentrates was almost equal in the hypophysectomized group and the intact group, but there was practically no response to normal female (with regular cycle) concentrates in hypophysectomized rats. The response to male gonadotrophic concentrates was definitely less marked in the hypophysectomized group than in the intact group. Similar results were obtained in hypophysectomized immature male rats, the response to post-menopausal and male concentrates being less in the hypophysectomized than in the intact animals. There was again no response to normal female concentrate in the hypophysectomized male group, but the crude gonadotrophic concentrate of an old man's urine was found more active than any other extract examined. However, it is obvious that the increase in weight of gonads, uterus and prostate of hypophysectomized rats, if any, calculated per cent increase over controls, was usually as great or even greater than that observed in intact rats.

Under the conditions of the present experiments, the morphological changes appear to be similar both in the intact and the hypophysectomized immature rats, in female extensive follicle stimulation, and in male advanced spermatogenesis being the most significant responses.

With the equivalent to 200 c.c. of postmenopausal and male urine, the enlargement of the ovaries was chiefly due to an increase in the number of developing follicles (Fig. 1 and 3). An ovarian response was observed within 120 hours, by which time a great number of mature uniform follicles developed, reaching the Graafian preparatory stage to ovulation. Even in primary follicles containing 7 or more layers of granulosa cells, the ova showed meiotic prophase with a very large nucleolus (Fig. 2 and 4). There were frequent mitotic divisions in theca externa and interna. No corpora lutea were found.

The enlargement of the testes was chiefly due to the development of seminiferous tubules with very active spermatogenesis. There was a layer of spermatogonial cells followed by a layer of spermatogonia in various stages of meiosis, followed again by spermatocytes showing the first stages of spermteleosis (Fig. 5 and 6). Spermatogenesis was never seen to end in production of fully differentiated sperms. No significant changes were observed in the interstitial cells of Leydig.

The uterus, prostate and seminal vesicles were less enlarged in comparison with the intact immature animals, but the uteri of the rats treated with postmenopausal concentrate were occasionally distended with fluid achieving sometimes a 10-fold size. In those animals the release of oestrogen was, in addition, indicated by the vaginal changes and smears. The immature vagina opened regularly 4 or 5 days after the first injection of concentrate with a pro-oestrus smear which changed to a cornified type.

When these data on the effect of urinary gonadotrophins on the reproductive organs of hypophysectomized immature rats were compared with previous results obtained on intact immature animals (Table II), it became clear that hypophysectomy affects the response quantitatively, but probably not qualitatively.

Other effects of crude urinary gonadotrophins.

Fig. 7 shows that the gonadotrophic concentrates had slight oxytocic activity. On several occasions there was an average oxytocic effect equivalent to about 3mU. Pitocin per 10 mg. of crude gonadotrophic powder, the average 24-hr.

TABLE II.—*Comparison of Effects of Urinary Gonadotrophins (≡ 200 c.c. of Urine) in Intact* and Hypophysectomized Immature Rats.*

Urinary concentrate.	Rats. No.	Rats. (I), (H)†	Necropsy: Mean weight of Body (g.).	Uterus (mg.).	Ovaries (mg.).	Increase over controls (per cent). Uterus.	Ovaries.
	20	(I)	48·7	18·8	10·6	—	—
	4	(H)	48·0	11·9	7·0	—	—
Postmenopausal women	16	(I)	44·5	80·8	41·8	329·7	294·8
	5	(H)	47·0	79·0	38·1	564·7	544·3
Old man	5	(H)	46·0	37·2	11·0	212·5	57·0
Young men	5	(I)	38·6	47·8	17·6	154·2	66·0
	5	(H)	46·0	27·8	10·2	133·6	45·7
Women with regular cycles	15	(I)	45·6	35·1	21·5	86·6	103·1
	4	(H)	49·0	16·7	7·8	40·3	11·4

				Testes (mg.).	Prostate (mg.).	Seminal vesicles (mg.).	Testes.	Prostate.	Seminal Vesicles.
	10	(I)	44·8	156·0	29·8	7·2	—	—	—
	5	(H)	44·8	107·2	11·1	5·5	—	—	—
Postmenopausal women	4	(I)	42·0	306·5	56·5	8·5	96·4	89·5	18·0
	5	(H)	42·6	271·0	39·3	8·9	152·7	245·0	61·8
Old man	5	(H)	49·8	340·0	45·9	7·4	217·1	313·5	34·5
Young men	5	(I)	36·0	240·0	57·6	12·6	53·8	73·1	75·0
	3	(H)	49·6	173·3	32·5	8·2	61·7	109·2	49·0
Woman with regular cycle	5	(H)	45·6	102·6	10·1	5·6	—	—	—

* Dekanski (1949*a*). † (I) Intact. (H) Hypophysectomized.

urinary specimen containing activity equivalent to between 70 to 90mU. of Pitocin. Crude gonadotrophic extracts contained some histamine on an average 0·2 μg. /372 mg. of powder (≡ 1400 c.c. of urine) which may affect the oxytocic response, but it is unlikely that the extract does contain enough histamine to have much effect on rats' blood pressure. The crude gonadotrophic suspension when injected intravenously into the rat caused usually a drop in blood pressure which was sometimes fatal, so that proper pressor assays were almost impossible. It seems that the crude gonadotrophic concentrates have no adrenocorticotrophic effect in those rats which survived the injection (Table III).

DISCUSSION.

Leonard and Smith (1934) were first to show that postmenopausal urinary extracts have a follicle-stimulating effect in both the intact and hypophysectomized rats. These results have been confirmed by Tyndale, Lewin and Smith

EXPLANATION OF PLATE.

FIG. 1.—The ovary of immature hypophysectomized rat injected with gonadotrophic concentrate equivalent to 200 c.c. of postmenopausal urine. (× 13.)

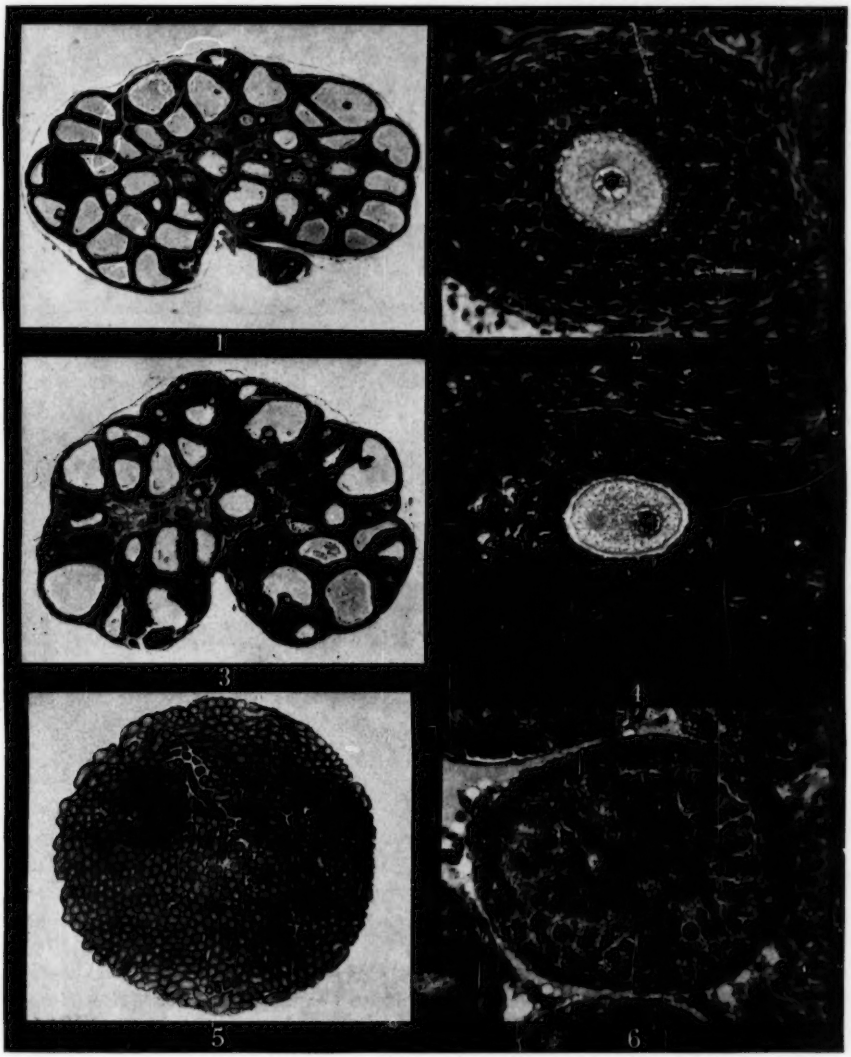
FIG. 2.—High power view (× 240) of section in Fig. 1.

FIG. 3.—The ovary of immature hypophysectomized rat injected with gonadotrophic concentrate equivalent to 200 c.c. of male urine (× 16.)

FIG. 4.—High power view (× 256) of section shown in Fig. 3.

FIG. 5.—The testis of immature hypophysectomized rat injected with gonadotrophic concentrate equivalent to 200 c.c. of urine from a 52 years old male. (× 11.)

FIG. 6.—High power view (× 246) of section shown in Fig 5.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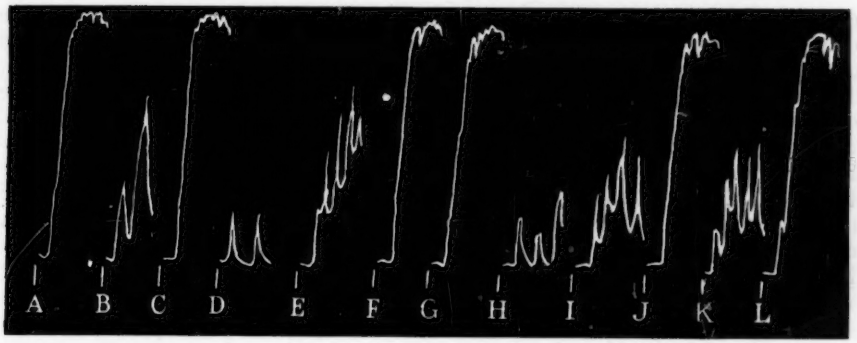

FIG. 7.—Oxytocic effect of crude gonadotrophic concentrates compared with that of Pitocin on the virgin guinea-pig's uterus. Ten-minute intervals. A, C, F, G, J and L = 0·005 i.u. Pitocin. B = 0·5 c.c. concentrate A (200 mg./10 c.c. normal saline). D = 0·2 c.c. concentrate A (200 mg./10 c.c. normal saline). E = 0·003 i.u. Pitocin. H = 0·2 c.c. concentrate B (200 mg./10 c.c. normal saline). I = 0·002 i.u Pitocin. K = 0·5 c.c. concentrate B (200 mg./10 c.c. normal saline).

TABLE III.—*Individual Estimates of Ascorbic Acid in the Adrenals of Hypophysectomized Rats Injected with Crude Urinary Gonadotrophins.*

Rat.		Right adrenal.		Dose* per 100 g. body weight	Left adrenal.		Difference.	
No.	Body wt. (g.).	Weight (mg.).	Ascorbic acid (μg./100 mg.).	(c.c.).	Weight (mg.).	Ascorbic acid (μg./100 mg.).	—	+
533	177	13·3	392	0·5				
538	230	12·2	339	0·5	16·6	347		8
540	348	15·8	408	1·0	18·3	390	18	
566	147	13·4	321	0·5	13·7	272	42	
570	119	16·6	367	0·25				
571	160	15·6	364	0·25	15·4	337	27	
572	130	15·0	382	0·25	14·9	370	12	
573	137	12·1	348	0·25				
576	161	11·8	395	0·5	14·7	374	21	
							Mean 18·7	

* Crude concentrate 1 c.c ≡ 72 c.c. of urine.

(1938), but not by Noble, Rowlands, Warwick and Williams (1939), who were unable to produce a response in the ovaries of hypophysectomized immature rats to the same dose of the postmenopausal extract. It seems that this discrepancy is due to the fact that the quantity of extract (≡ 19 c.c. of urine) was too small to have much effect on hypophysectomized immature rats.

The results presented here suggest that the injection of crude postmenopausal gonadotrophic concentrates produced similar follicle stimulation (mitosis promoting effect) in both the intact and hypophysectomized immature rats when given equally large doses of those concentrates for the same time. The response of the ovaries to the male urinary concentrates was less pronounced and that to the normal female (with regular cycle) almost undetectable. The crude urinary

gonadotrophic concentrates of postmenopausal and male urine produced a definite response in the testes of both the intact and hypophysectomized immature rats, but again those obtained from the young woman had no action. This absence of significant effect does not necessarily suggest a qualitative difference in the actions of different gonadotrophic concentrates of the normal urines. It is probably due to the fact that the male and especially the normal female concentrates contained less of the active principles, and that in hypophysectomized animals these concentrates may induce only follicular growth and spermatogenesis until very high quantities are used. It seems, therefore, that in experiments of the type discussed here, the difference in response in intact and hypophysectomized immature rats to injections of crude urinary gonadotrophins was quantitative rather than qualitative.

No adrenocorticotrophic or pressor activity was detected, but the concentrates were too toxic when injected intravenously into the rat, so that the results obtained are uncertain and cannot be usefully discussed.

SUMMARY.

The urinary gonadotrophic hormones concentrated by means of kaolin were injected subcutaneously into immature hypophysectomized rats. The difference in the response to large doses ($\equiv$ 200 c.c. of urine) in immature intact and hypophysectomized rats seems to be quantitative rather than qualitative.

I wish to thank Professor J. H. Gaddum and Dr. Max Reiss for their valuable help and advice, and also Organon Laboratories, Ltd., for providing facilities for this work.

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